Lymphocyte Activation Gene-3 – the expression and function in the immune system

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ABBREVIATIONS

Ab	antibody
Ag	antigen
ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
BCR	B cell receptor
BSA	bovine serum albumin
CD40L	CD40 ligand
CFSE	carboxyfluorescein diacetate, succinimidyl ester
CγC	common gamma chain
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DNA	deoxyribonucleic acid
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FDC	follicular dendritic cell
GC	germinal center
GFP	green fluorescent protein
HA	hemaglutinin
H chain	heavy chain
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
int	intermediate
kDa	kilodalton
KO	knock-out
LAG-3	lymphocyte activation gene-3
LN	lymph node
LPS	lipopolysaccharide
mAb	monoclonal antibody

MHC	major histocompatibility complex
MT promoter	metallothionein promoter
MZ	mariginal zone
neg	negative
NFAT	nuclear factor of activated T cells
NF-kB	nuclear factor kappa B
NK	natural killer
o/n	over-night
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PHA	phytohaemagglutinin
poly(I)-poly(C)	polyriboinosinic acid polyribocytidylic acid
RAG	recombination activating gene
RBC	red blood cell
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription - polymerase chain reaction
SDS	sodium dodecyl sulfate
SEB	staphylococcal enterotoxin B
sLAG-3	soluble LAG-3
TCR	T cell receptor
TD	T cell dependent
TI	T cell independent
Th	helper T lymphocyte
Treg	regulatory T cell
TLR	Toll like receptor
TNFα	tumor necrosis factor α
TNFβ	tumor necrosis factor β
WT	wild-type

SUMMARY

Lymphocyte activation gene-3 (LAG-3) is the structural homologue of a well-known TCR co-receptor CD4. Recently, it has been suggested that LAG-3 might play a role in modulation of immune response, by being a negative regulator of T cell activation.

To further characterize its expression and function in the immune system, monoclonal Abs were generated against mouse LAG-3. The availability of different mAbs allowed the identification of LAG-3 on various immune subpopulations.

The main finding of this work is that, unlike previously thought, LAG-3 expression is not limited to activated T and NK cells, but can also be induced on B cells and DCs. B cells were found to express LAG-3 in a T cell dependent manner; LAG-3 was expressed on B cells in the presence of activated T cells, but not upon stimulation with thymus independent stimuli like CpG or LPS. Furthermore, requirements for LAG-3 induction on B cells were defined; this event requires B cells proliferation and is mediated by a soluble factor released by activated T cells.

Among DCs, two populations, CD8- ('myeloid') and plasmacytoid DCs, expressed high levels of surface LAG-3 upon stimulation with LPS and CpGs, respectively.

To evaluate the potential role of LAG-3 in APC function, LAG-3 deficient and OT-II transgenic mice were used. Antigen presentation assay showed, that LAG-3 presence on the surface of APC can affect T cell responses. T cells stimulated with OVA peptide presented by LAG-3 deficient B cells proliferated poorly compared to T cell stimulated with OVA-pulsed LAG-3 positive B cells. This finding suggests a novel co-stimulatory function of LAG-3 on APCs.

In addition, experiments re-evaluating published data concerning the role of LAG-3 on T cells were performed. In agreement with previous reports, the ectopic expression of LAG-3 on the surface of T cell line exerted an inhibitory effect on T cell activation induced by relevant antigen. However, in disagreement with recent report (by Huang et al. 2004), the standard regulatory cell assays performed with normal as well as LAG-3 deficient CD4+CD25+ cells, suggested no involvement of LAG-3 in regulatory T cell function.

Furthermore, the experiments demonstrating the surface expression on murine CD4 and CD8 T cells *in vivo* are presented. LAG-3 was identified on homeostatically expanding T cells after transfer into lymphopenic mice.

To investigate structural aspects of the LAG-3 molecule, soluble LAG-3 (sLAG-3), composed of four extracellular domains was produced. For this purpose *Drosophila* expression system was employed. The conditions were developed for production of two forms of sLAG-3, the original glycosylated form and its non-glycosylated version. Biochemical analysis of soluble protein showed that in solution LAG-3 forms stable dimers, which suggests the presence of such dimers on cell surface. Because heterogenous glycosylation of WT form likely prevents protein crystallization, attempts are now made to crystallize non-glycosylated form of sLAG-3 alone or in complex with its ligand, MHC class II. The detailed structural information would significantly enhance our knowledge about LAG-3 and allow for rational drug design, if desired.

1. INTRODUCTION

1.1 The Immune System

The immune system of our body is responsible for protection against the myriad of potentially pathogenic microorganisms that inhabit the world we live in. It is an organization of cells and molecules with specialized roles, which work around the clock in many different ways.

In an adult organism, the cells of the immune system originate in the bone marrow, where many of them also mature. Others, like T cell progenitors, need to migrate to the thymus to undergo maturation. They then leave these primary lymphoid organs to patrol the tissues by circulating in the blood and in a specialized structure called the lymphatic system. Organized lymphoid tissues such as lymph nodes (LN) and spleen as well as Peyer's patches of the intestine and tonsils, called secondary lymphoid organs, are the place where the initiation of the adaptive immune responses occurs. Various macromolecules or any foreign substance (such as virus, bacteria or protein) able to elicit immune responses in the body are termed antigens. The responses to antigens present in the lymph are mounted in lymph nodes while the spleen is a site of the induction of immune responses to antigens in the blood stream.

There are two fundamentally different arms of the immune system: the innate and the adaptive responses. Both are tightly linked and work in concert to eliminate the pathogen. The innate immunity is the less specific component, which provides the first line of defense against infections. Most of its 'weapons' are present before the onset of infection so it can act very fast. It has the property of pattern recognition, an ability to recognize a class of molecules unique to microbes. Phagocytic cells (such as macrophages and neutrophils), anatomic barriers (such as skin and mucous membranes), physiologic factors (temperature, low pH, chemical mediators) and a variety of antimicrobial compounds synthesized by the host, all play important roles in innate immunity.

The adaptive immunity is aimed at the particular antigen, but the primary response takes longer (five to six days) to develop. It involves the proliferation of antigen-specific B and T cells, which occurs when the surface receptors of these cells recognize the relevant antigen. Specialized cells display the antigen to lymphocytes and collaborate with them in the response to antigen. The adaptive immunity possesses the remarkable property of memory manifested by faster and stronger attack upon secondary encounter with the antigen that induced the primary response. It is also capable of discriminating self from non-self (the foreign particle) or altered-self (a cell infected by a virus or a cancerous cell), which prevents it to be aggressive against the own tissue.

1.1.1 Players of adaptive immunity

The key players of adaptive immunity are B cells and T cells. For a proper immune response, however, they need assistance from Antigen Presenting Cells (APCs).
B cells produce Immunoglobulins (Igs, also called antibodies), and manage humoral (that is pertaining to extracellular fluid) immunity, while T cells are non antibody-producing lymphocytes and constitute the basis of cell-mediated immunity.

1.1.1.1 B lymphocytes

B lymphocytes are generated in the bone marrow and migrate to the periphery as short-lived and functionally immature cells. Through the blood stream they reach the spleen where they develop into long-lived mature B cells (Carsetti et al., 2004; Rolink et al., 2004). Each B cell expresses a unique antigen binding receptor (B- cell receptor or BCR) on their surface. BCR is a membrane-bound Ig molecule consisting of two identical heavy (H) chains and two identical light (L) chains that are held together by disulfide bonds and non-covalent interactions. It is associated with two trans-membrane proteins Ig-α and Ig-β, which are crucial for triggering downstream signaling pathways. Each chain of the antibody folds into a series of compact domains of similar structure, the so-called immunoglobulin fold (Ig fold). This structure of approximately 110 amino acids consists of a “sandwich” of two β-pleated sheets, each containing anti/parallel β strands, which are connected by loops of various lengths (Frazer and Capra, 1999).

When a naïve B cell first encounters its specific antigen, the binding of the antigen to the BCR causes the cell to divide rapidly and undergo differentiation into antibody-secreting B cells (also called plasma cells) and memory B cells.

Depending on the nature of antigen, the B-cell activation proceeds by two different routes: thymus dependent (TD) or thymus independent (TI). TD antibody responses to protein antigens require antigen-specific T cell help in the form of surface molecules' interactions as well as secretion of soluble factors. Microbial antigens with repetitive determinants (like lipopolysaccharides, polysaccharides, lipoprotein A) can stimulate naïve B cells on their own and thus are thymus independent. The TI antigens can be further divided into two groups: TI-2 antigens, which are multivalent molecules able to crosslink BCR, and TI-1 antigens activating B cells independently of BCR, acting through Toll-like receptors (TLRs). TI-1 antigens, for example LPS, can cause the polyclonal proliferation and differentiation of many B cells regardless of their antigenic specificity.

Mature B cells can be subdivided into at least 3 groups: B2 B cells, B1 B cells and the mariginal zone B cells;

- B2 B cells

B2 B cells, the conventional B cells, constitute the majority of B cells found in spleen and lymph nodes. This pool of B cells is constantly replenished by differentiation of progenitor cells in the bone marrow (Rolink et al., 2004). B2 B cells produce high affinity antibodies after they have been stimulated, expanded and selected in germinal centers in the presence of T cell help (Carsetti et al., 2004; Fagarasan et al., 2000). They can give rise to memory B cells as well as plasma cells.

- B1 B cells

B1 cells arise before B2 cells and compose about 5% of B cell population. B1 B cells, originally defined by the surface expression of CD5 molecule and high levels of IgM, have a capacity for self-renewal (Herzenberg et al., 1986; Marcos et al., 1989). They are dominant in the peritoneal and pleural cavities, rare in the spleen and extremely rare in lymph nodes (Forster et al., 1991; Hayakawa et al., 1985; Herzenberg and Kantor, 1993). They exhibit different variable heavy chain repertoire and Ig specificities, compared to

B2 cells (Fagarasan et al., 2000). B1 B cells are the source of so-called natural antibodies that are pre-existing or produced during early phase of infection. These antibodies, that appear to be produced in a T-cell independent manner (Fagarasan and Honjo, 2000), tend to have a low affinity and broad specificities (Carsetti et al., 2004; Lalor and Morahan, 1990). B1 B cell subpopulation recognizes common bacterial pathogens and plays an important role in mucosal immunity. But the antibodies produced by B1 B cells often react to self-antigens (Berland and Wortis, 2002; Fagarasan et al., 2000).

- mariginal zone B cells

Mariginal zone (MZ) B cells are the B cells residing in the splenic mariginal zone, the junction of white and red pulp. They have a distinct surface phenotype, including higher expression of complement receptors and IgM (Oliver et al., 1997). MZ B cells play an important role in T-independent antibody responses (Fagarasan and Honjo, 2000). They more rapidly express activation markers and co-stimulatory molecules than conventional B cells. In some aspects they resemble peritoneal B1 cells. They participate very early in immune responses and are very sensitive to LPS stimulation, which can induce their rapid differentiation into plasma cells (Martin and Kearney, 2000; Oliver et al., 1997).

1.1.1.2. T lymphocytes

T cells also derive from cells of the bone marrow, but their precursors migrate to the thymus gland to mature. During maturation a T cell comes to express the T cell receptor (TCR) on its surface. And it is in the thymus, where the T cells-to-be learn how to distinguish self from non-self, and where the useless cells (the cells not able to recognize anything) are eliminated (von Boehmer et al., 1989).

Unlike BCRs, TCRs are produced only as transmembrane molecules. A TCR unit consists of α/β or γ/δ heterodimer in complex with CD3 components. Only as such it can play its role in T cell activation. The antigen-binding site is formed by the combination of N-terminal Ig-like domains of the α/β or γ/δ dimers, while C-terminal Ig-like domains are constant for each type of TCR chain. CD3 complex consists of the CD3 γ, δ and ε chains associated with a homodimer of ζ chains or a heterodimer of ζ and η chains and provides the connection to the intracellular signalling machinery.

In contrast to BCR, which can recognize antigen alone, αβ TCR can recognize it only in the form of antigenic peptides that are bound to the major histocompatibility (MHC) molecules, polymorphic glycoproteins found on cell membrane. The ability of T cells to recognize antigen exclusively in the context of MHC is called MHC restriction. When a T cell encounters its specific antigen presented by MHC, it proliferates and differentiates into effector T cells and memory T cells.

– αβ T cells

There are two well defined subpopulations of αβ T cells: helper T cells and cytotoxic cells and two less well defined subpopulations of regulatory T cells and natural killer T cells.

- Helper T cells

Helper T cells (Th) express the CD4 glycoprotein surface molecule and thus are also known as CD4 T cells. CD4 is an accessory molecule (often called co-receptor) facilitating interactions of T cells with MHC class II, due to its specific affinity for MHC class II molecules. CD4 T cells can recognize the antigen only when it is presented in the MHC class II context (in other words, they are MHC class II restricted). The antigenic peptides presented on MHC class II derive mainly from exogenous proteins acquired by endocytosis.

Besides stabilizing T cell- MHC class II bearing cell interactions, CD4 molecule may mediate signal transduction, thereby promoting the subsequent functional responses. CD4 T cell activation is tightly controlled; by MHC class II restriction and by limiting the expression of MHC class II on the cells of the body. MHC class II is expressed only on specialized cells, called antigen presenting cells (APCs).

After a T helper cell recognizes and interacts with peptide-MHC class II complex it gets activated and gives rise to a clone of effector cells that secrete various cytokines. These cytokines play an important role in activating other cells that participate in the response. They are needed for optimal protection and they may also reduce allergic and autoimmune responses (Delves and Roitt, 2000; Gately et al., 1998; Grunig et al., 1997).

The differences in pattern of cytokines produced by CD4 T cells result in distinct responses. Based on the cytokines they produce, CD4 effector T cells fall into two functional categories: Th1 and Th2. The Th1 cells produce Interleukin-2 (IL-2), Interferon-γ (IFN-γ) and Tumor necrosis factor β (TNFβ) that have an effect on the production of opsonizing and complement-fixing antibodies, activate macrophages, support inflammation and activate T cells. Th2 response, on the other hand, involves the production of IL-4, IL-5, IL-6, IL-9 and IL-13 and induces strong antibody responses, favors eosinophil differentiation and activation, but inhibits phagocytic cells (Abbas et al., 1996; Mosmann et al., 1986; Romagnani, 2000). But more cytokine patterns are possible; T cells expressing Th1 as well as Th2 cytokines have been designated as type 0 (Th0), while T cells producing high amounts of TGFβ have been termed as type 3 (Th3)(reviewed in (Romagnani, 2000).

- Cytotoxic T cells

Cytotoxic T lymphocytes (CTLs) express the CD8 co-receptor in the form of αβ heterodimer or αα homodimer, and therefore are also called CD8 T cells. CD8 molecule, by binding to MHC class I, serves as a cell-cell adhesion molecule and as enhancer of signal transduction. CD8 T cells are MHC class I restricted and since MHC class I, unlike MHC class II, is expressed virtually on all nucleated cells of the body, any cell can mark itself as a CD8 cell target. These cells display peptides derived from intracellular proteins on their surface in the context of MHC class I all the time. A cell becomes a target when a foreign, for example viral, peptide is presented on the cell surface. Under the influence of Th1 derived cytokines, a CD8 T cell recognizing an antigen- MHC class I complex proliferates and differentiates into an effector cell. CTL can kill the infected cell in at least two different ways; (1) perforin/granzyme pathway or (2) Fas receptor-mediated pathway (Delves and Roitt, 2000; Kagi et al., 1994).

- Regulatory T cells

Regulatory T cells (T regs) represent the minor subpopulation of T cells that are thought to perform a specialized role in controlling both the innate and adaptive immunity (Maloy and Powrie, 2001; Maloy et al., 2003; Sakaguchi et al., 2001). They are able to actively

suppress self-reactivity of lymphocytes or their excessive response to pathogen, both of which can cause damage to the host (Sakaguchi et al., 2001). Specifically, T regs include those that are able to suppress naïve T-cell proliferation *in vitro* and to control CD4+ and CD8+ T cell numbers *in vivo* in lymphopenic host (O'Garra and Vieira, 2004).

There is no clear-cut definition of suppressor cells, but in recent years the evidence has accumulated that CD4+ CD25+ cells can perform such a function. The CD4+CD25+ subset of T cells represents 5-10% of the CD4+ T lymphocytes in healthy human and mice. Their development and function depends on the forkhead/winged transcription factor Foxp3. Foxp3 is restricted to Tregs and is expressed in these cells irrespective of their state of activation. Moreover, it was demonstrated that ectopic expression of Foxp3 confers suppressor function on peripheral CD4+CD25- T cells (Fontenot et al., 2003; Hori et al., 2003). In contrast, the induction of CD25 expression by stimulating CD4+ CD25- cells with anti-CD3, mitogens, or specific antigen does not render the stimulated cells suppressive (Shevach et al., 2001).

T regs require TCR triggering in order to be suppressive *in vitro*. But, although their activation is antigen specific, once activated these cells inhibit both CD4+ and CD8+ responses in an antigen non-specific manner (Takahashi et al., 1998; Thornton and Shevach, 2000). Interestingly, the suppression of T cell proliferation by T regs does not involve killing of the responder cells and is mediated through a cell contact dependent mechanism (Maloy and Powrie, 2001; Shevach et al., 2001). The cell surface molecules involved in T reg - T responder interaction, however, remain to be characterized. Furthermore, regulatory cells do not prevent the initial steps of responder T cell activation (the expression of early activation markers is not affected), but the activated cells fail to proliferate due to cell cycle arrest at the G0/G1 stage (Thornton and Shevach, 2000).

- NK T cells

Natural killer T cells (NK T cells) are a distinct population with certain phenotypic characteristics of NK cells (see below), but also express CD4 and intermediate levels of TCR with a highly restricted repertoire of specificities. These cells recognize antigen

presented by the non-classic MHC molecule CD1 and may have an immunoregulatory role, because they can secrete IL-4 and IFN-γ (Burdin and Kronenberg, 1999).

– γδ T cells

In human and mice, γδ T cells represent a small percentage (less than 5%) of the lymphocytes in the thymus and peripheral lymphoid organs such as lymph nodes and spleen and in circulation), but they are a major population (up to 70%) in the mucosal epithelia (Hayday et al., 2000). During development in the thymus γδ T cells appear before αβ T cells. They are recruited and expand in response to various infections in humans and rodents (Bukowski et al., 1999). The vast majority of γδ T cells are CD4 and CD8 negative (Fisher and Ceredig, 1991). γδ TCRs are of limited diversity and can recognize unprocessed ligands and even nonproteinacious phospholigands directly, without a requirement for presentation on MHC (Kaufmann, 1996). The role of γδ cells in the immune system, however, is still enigmatic.

1.1.1.3. Antigen Presenting Cells

Antigen Presenting Cells (APCs) are specialized cells characterized by the expression of MHC class II on their surface and the ability to deliver co-stimulatory signals required for T cell activation. APCs include Dendritic Cells (DCs), macrophages and B cells.

T cells require two types of signals from APC for activation and subsequent differentiation into an effector cell. First one is an antigen-specific signal provided by interactions between the TCR and antigenic peptide presented by MHC molecules on APC. The second signal (co-stimulation) is antigen-independent and mediated by engagement of the T cell surface molecule CD28 with the members of B7 family (like B7-1/CD80; B7-2/CD86) on the APC.

Molecular events leading to T cell activation occur in a so-called immunological synapse between T cell and APC (Figure 1.1). This synapse has a complex supramolecular structure containing many different cell surface proteins whose individual functions are not yet completely understood.

- Dendritic Cells

Dendritic cells are 'professional' antigen presenting cells, i.e. they are specialized for the uptake, processing, transport and presentation of antigens to T cells. They are sparsely distributed in non-lymphoid tissue, but have a distinct migratory capability (Hart, 1997; Matzinger, 1994; Steinman, 1991). Recruitment of DCs from the bone marrow into peripheral non-lymphoid tissues as well as migration of mature DCs from the periphery into the lymphoid tissues are coordinated by chemotactic cytokines (chemokines). Chemokines interact with corresponding receptors on DCs (Hackstein and Thomson, 2004).

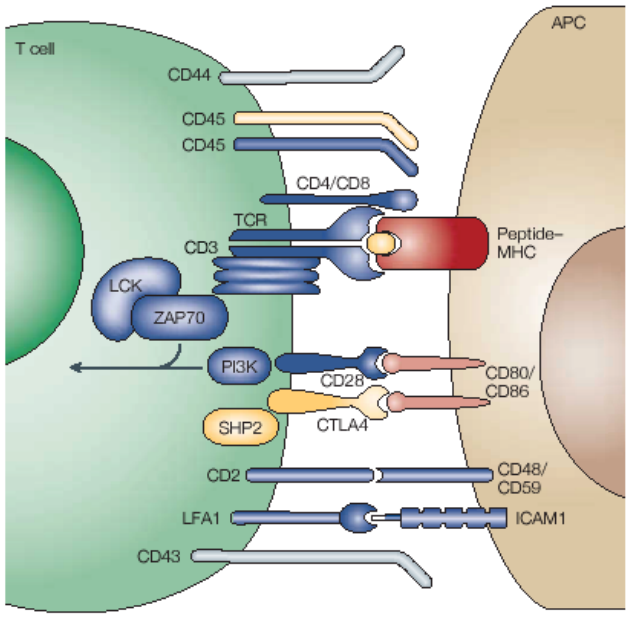


Figure 1.1 Overview of a mature T cells synapse. The key ligand pairs and signaling molecules that are involved in T cell recognition are shown. The MHC molecule presenting the stimulatory peptide is shown in red, activating molecules are blue, B7 family surface molecules in pink, inhibitory molecules are yellow and molecules that are not contributing to signaling are shown in grey (Huppa and Davis, 2003).

DCs arise from the bone marrow progenitor cells or blood monocytes and differentiate into immature DCs (Banchereau et al., 2000). At 'immature ' stage of development DCs act as sentinels in peripheral tissues or circulate in blood continuously sampling the environment. They are already capable of antigen uptake and processing, but yet are unable to activate T cells due to low level of expression of surface MHC class II

molecules and lack of expression of co-stimulatory molecules (Shortman and Liu, 2002) ((Hackstein and Thomson, 2004). At the encounter with the 'danger' signal (for example microbial products or tissue damage), the antigen processed and displayed on the DC surface as peptide MHC complex is stabilized by decreasing MHC class II turnover, and the maturation and concomitant migration of DCs to LN takes place. On the way, maturing DCs down-regulate endocytic activity and up-regulate the co-stimulatory molecules that are required for effective interactions with T cells (like CD40, CD80 and CD86) (Banchereau et al., 2000; Cella et al., 1997). This process is accompanied by the production of inflammatory cytokines, such as IL-12 and TNFα (Hackstein and Thomson, 2004). DC maturation can be elicited by numerous stimuli; (1) by endogenous factors that are released by necrotic cells (for example, heat shock proteins), (2) by exogenous microbial products including lipopolysaccharide, LPS, peptidoglycans or CpG rich DNA, that bind to Toll-like receptors (TLRs) or other pattern recognition receptors, (3) by pro-inflammatory cytokines that are produced by bystander cells (like TNFα), and (4) by activated T cells expressing ligands for co-stimulatory molecules, like CD40 ligand (CD40 L/ CD154) (reviewed in (Hackstein and Thomson, 2004)).

After migration to the LNs, the mature DCs efficiently trigger an immune response by activating T cell with the receptor specific for the foreign peptide-MHC complex present on the DC surface (Shortman and Liu, 2002). In addition to the interactions with T cells, DCs interact with other cell types including B cells and NK cells present in the lymphoid organs. Alternatively, DCs might also remain in peripheral tissues and act as mediators of inflammation.

Besides the insult-induced migration, DCs show continuous low-level migration from periphery to LNs. These DCs are not activated and, since immature DCs trigger T cell anergy rather than activation, they probably contribute to the peripheral tolerance of T cells (Inaba et al., 1998; Probst et al., 2003).

DCs are not a homogenous population, but comprise of at least five different subtypes related to their origin, differentiation state and their specific location. The functional significance of this heterogeneity is currently under intense study.

1.1.1.4. Natural Killer Cells

Natural Killer (NK) cells are another class of lymphocytes that posses a potent cytolytic activity. Because they lack antigen specific receptors, NK are defined as a component of the innate immune system, but they were also shown to participate directly in adaptive immune responses, mainly by interacting with dendritic cells (Raulet, 2004). Also, NK cells produce a number of cytokines, especially IFN-γ, and therefore are important for immune regulation and influence both innate and adaptive immunity.

NK cells destroy infected and malignant cells. To identify target cells NK cells use less specific broad-spectrum receptors (Wang et al., 1997), one kind of which are the Fc receptors. Fc receptors on the surface of NK cells link them to antibody (IgG) coated target cells, which are then killed by a process called antibody-dependent cellular cytotoxicity (ADCC). The second system of recognition involves the killer-activating and killer-inhibitory receptors. The killer-activating receptors recognize a number of different molecules present on the surface of nucleated cells, whereas the killer-inhibitory receptors recognize MHC class I molecules (Moretta et al., 1997) (Lanier, 1998). If the activating receptors are engaged, the NK cell becomes cytotoxic, but this signal is normally overridden by an inhibitory receptor upon recognition of MHC class I molecule. Cells without MHC class I on their surface (the loss of which can be a consequence of viral infection or malignant transformation) are unable to activate inhibitory receptors and thus are killed (Moretta and Moretta, 2004).

1.1.2. Lymphocyte homeostasis

In an adult mammal the total number of lymphocytes remains steady throughout an animal's life, thanks to a "return tendency, due to a density-dependent process to maintain a stationary distribution of population densities" (Hanski, 1999), usually referred to as homeostasis (Freitas and Rocha, 2000; Marrack et al., 2000).

New lymphocytes are continually being produced in primary and secondary lymphoid organs, and old or useless cells are continually being eliminated (Rathmell, 2004). Because the potential to produce new cells exceeds the number needed to replenish peripheral pools, the new cells must compete with resident ones for survival signals and

resources (Freitas and Rocha, 2000). Since each lymphocyte has a different Ag–binding receptor, the decision about which cell survives and which dies shapes the repertoire of the immunocompetent cells. Thus the homeostatic control of cell number provides one of the mechanisms of lymphocyte selection.

The homeostatic control of cell number is also important after the antigenic challenge. After an infection, there are bursts of cellular proliferation, but once the infectious agent is eliminated, the number of cells returns to the state before the infection (Gaudin et al., 2004). This is a result of death of most of the activated cells, with a small fraction surviving as memory cells. The number of long-lived memory cells also must be limited in order to preserve the diversity of repertoire (Grossman et al., 2004).

The mechanisms of such immunoregulation are complex and act at different stages of the immune response. Both membrane-bound molecules (receptors, co-receptors) and soluble factors (cytokines) are involved in homeostatic regulation.

1.1.3. T cell responses

There are 4 steps to the T cell response; activation - during which the cell receives 'instructions' in the form of antigenic stimulation, followed by a burst-like expansion (the numbers of activated cells increase rapidly), which after reaching a plateau (upon antigen clearance) eventually decreases - contraction, a process accompanied by a massive cell death and memory cell development.

The engagement of TCRs and co-stimulatory proteins on the surface of T cells leads to activation. Signal transduction pathways associated with T cell activation include cascades of kinase signalling, Ca2+ -mediated and small G -protein mediated pathways (Lin and Weiss, 2001). The combination of those initiates proliferation of cells and makes them receptive to stimulatory signals delivered through IL-2 receptor. They also induce production of cytokines such as IL-2 (June et al., 1990), which act in an autocrine fashion to support further division of activated cells (Marrack et al., 2000).

The activated T cells differentiate into a variety of cell fates. For example, CD8 T cells become CTLs, capable of killing virus-infected cells. CD4 T cells polarize toward Th1 or Th2 and mediate protection from intracellular or extracellular pathogens.

Yet, activated T cells are short lived. The process of elimination of effector cells is driven by several different pathways, among them, two well-known pathways leading to apoptosis: Fas-FasL (Ettinger et al., 1995; Van Parijs and Abbas, 1996) or tumor necrosis factor (TNF) receptor (Sytwu et al., 1996; Zheng et al., 1995) and another way triggered by reactive oxygen species produced within the activated cell (Hildeman et al., 1999). Other control mechanisms include (1) competition for growth and viability signals (the production of cytokines by activated lymphocytes and APCs decreases once antigen is eliminated)(Ahmed and Gray, 1996) (Freitas and Rocha, 2000), (2) competition for access to APCs or peptide-MHC ligands (Grossman et al., 2004), (3) elimination of APC by cytotoxic T cells (Wong and Pamer, 2003), and (4) suppression by specialized regulatory cells (Maloy and Powrie, 2001; Shevach, 2000).

Interestingly, the rapid disappearance of activated T cells during the contraction phase does not affect the number of naïve nor memory cell pools (Marrack et al., 2000).

Only 5-10% of antigen specific population survives the contraction phase of primary immune response and is maintained indefinitely for years (Masopust et al., 2004). These memory cells provide immediate protection and generate more rapid and effective responses when again encountering the antigen (Ahmed and Gray, 1996; Dutton et al., 1998; Zinkernagel et al., 1996).

The regulation of T cell activation and effector T cell expansion during immune responses is essential for maintenance of T cell homeostasis.

Many surface molecules have been identified that participate in the activation of a T cell; such as CD28, OX 40, 4-1 BB, LFA-1 and CD2 (reviewed by (Marrack et al., 2000) (Watts and DeBenedette, 1999)), but few are known that are important for termination of activatory signals.

A well-documented example of a molecule important for down-regulation of T cell responses is cytotoxic T lymphocyte antigen 4 (CTLA-4/CD152)(Krummel and Allison, 1995; Walunas et al., 1994). This immunoglobulin superfamily member is an inhibitor of the CD28- B7 co-stimulation pathway (Oosterwegel et al., 1999). While CD28 is constitutively expressed on both activated and naïve T cells (Gross et al., 1990), CTLA-4 is absent in naïve T cells, but is readily detectable on the cell surface within 24 hrs of stimulation, with maximal expression at 2-3 days post-stimulation (Lindsten et al., 1993;

Linsley et al., 1992) (Linsley et al., 1992). After appearing on the cell surface CTLA-4 competes favourably with its homologue CD28 for B7 molecules on APCs, because of a significantly higher avidity for B7 (Greene et al., 1996; van der Merwe et al., 1997).
CTLA-4 deficient mice show a strong phenotype. Their T cells proliferate massively, which leads to enlarged lymph nodes and spleen, multiorgan lymphocytic infiltration and death within 3 to 4 weeks after birth, showing that indeed CTLA-4 is vital for lymphocyte homeostasis (Tivol et al., 1995; Waterhouse et al., 1995).

Recently, another molecule was identified and suggested to play an inhibitory role in T cell activation - Lymphocyte Activation Gene-3. Characterization of LAG-3 surface molecule is the topic of this thesis (for detailed information on Lymphocyte Activation Gene-3 see section 1.2).

1.1.4. T cell-B cell collaboration in B cell responses

The cooperation among the components of the adaptive immunity is absolutely required for efficient antibody production in response to antigenic challenge.
Ag-specific B cell - T cell interactions occur within defined areas of secondary lymphoid organs, namely the edges of the T and B cell zones (Garside et al., 1998; Goodnow, 1997). In the white pulp of the spleen, T cells are arranged in the T-cell area around a central arteriola and make up the periarterial lymphatic sheath (PALS). B cells collect in the primary follicles surrounding PALS and in the mariginal zone, external to the follicles (Timens, 1991; Witmer and Steinman, 1984).
According to the two-phase B cell activation model it is the second phase of the B cell activation cascade, characterized by the germinal center reaction, that is T cell dependent. The initial Ag-specific B cell activation is induced in secondary lymphoid tissues, by binding of antigens to the BCR. At the same time as initial activation and clonal expansion of B cells takes place, T cell activation and expansion occurs driven by their interactions with professional APC, that provide all signals necessary for T cell stimulation (Baumgarth, 2000) (Figure 1.2, Phase 1).
Because DCs very efficiently focus and transport antigens from peripheral tissues to T cells zones of secondary lymphoid tissues, and are potent activators of T cells (Banchereau and Steinman, 1998), it is generally thought that T cell are initially activated

by DCs in response to tissue localized antigens, while splenic B cells may be the initiating APC for blood-borne antigens (Mills and Cambier 2003).

Naïve B cells do not collaborate effectively with T cells, but antigen encounter prepares them to activate T cells and to receive T cell-derived help signals. Within minutes after BCR triggering, BCR-antigen complex is internalized and antigenic peptide presented in the MHC class II context (Siemasko and Clark, 2001). Concomitantly, B cells increase the expression of cell-surface molecules important for productive interaction with T cells, including CD80/CD86 (B7.1/B7.2) and MHC class II. Antigen-specific B cells then move to the edge of the follicles and undergo cognate stimulatory interactions with antigen activated T cells. The B cells subsequently become primary antibody forming cells (AFCs) or precursors destined for the germinal center (GC) (Figure 1.2, Phase II) (Manser, 2004)

Besides Ag peptide-MHC complex – TCR interplay, several membrane molecules are involved in the generation of TD humoral responses, of which a TNF receptor family member - CD40 interaction with its ligand-CD40L (CD154) plays the pivotal role. CD40 is constitutively expressed on mature B cells and upon aggregation triggers a number of intracellular signaling pathways leading to clonal expansion, germinal center formation, affinity maturation, and generation of long-lived plasma cells (Bishop and Hostager, 2001; Garside et al., 1998). Interestingly, the CD40L is expressed at high levels primarily on the surface of activated T cells particularly following CD80/86 mediated CD28 co-receptor signaling. This demonstrates, that CD40 signal transduction is tightly regulated by ligand availability and normally occurs after B cells have encountered cognate antigens and contacted antigen-specific CD4+ T cells (Mills and Cambier, 2003). Activated B cells can reciprocally trigger cognate T cells to clonally expand and differentiate (Quezada et al., 2004). It was shown that the induction of optimal levels of T cell priming to a protein Ag requires the involvement of Ag-specific B cells (Constant, 1999).

Although contact-mediated signals are critical for productive T-B cell collaborations, T cells produce soluble factors that are important for B cell differentiation as well. One of the best characterized of these cytokines is IL-4. It enhances B cell survival, primes them for MHC class II signaling, increases expression of co-stimulatory molecules and

prepares them for maturation steps (Nelms et al., 1999). Other T cell-derived cytokines involved in B cell differentiation include IL-2, IL-3, IL-6 and IL-10 (Mills and Cambier, 2003) (Coffman et al., 1988). The B cell responsiveness to the cytokine signals, however, was shown to occur only after stimulation of their cell membrane molecules by activated T cells (Bartlett et al., 1990).

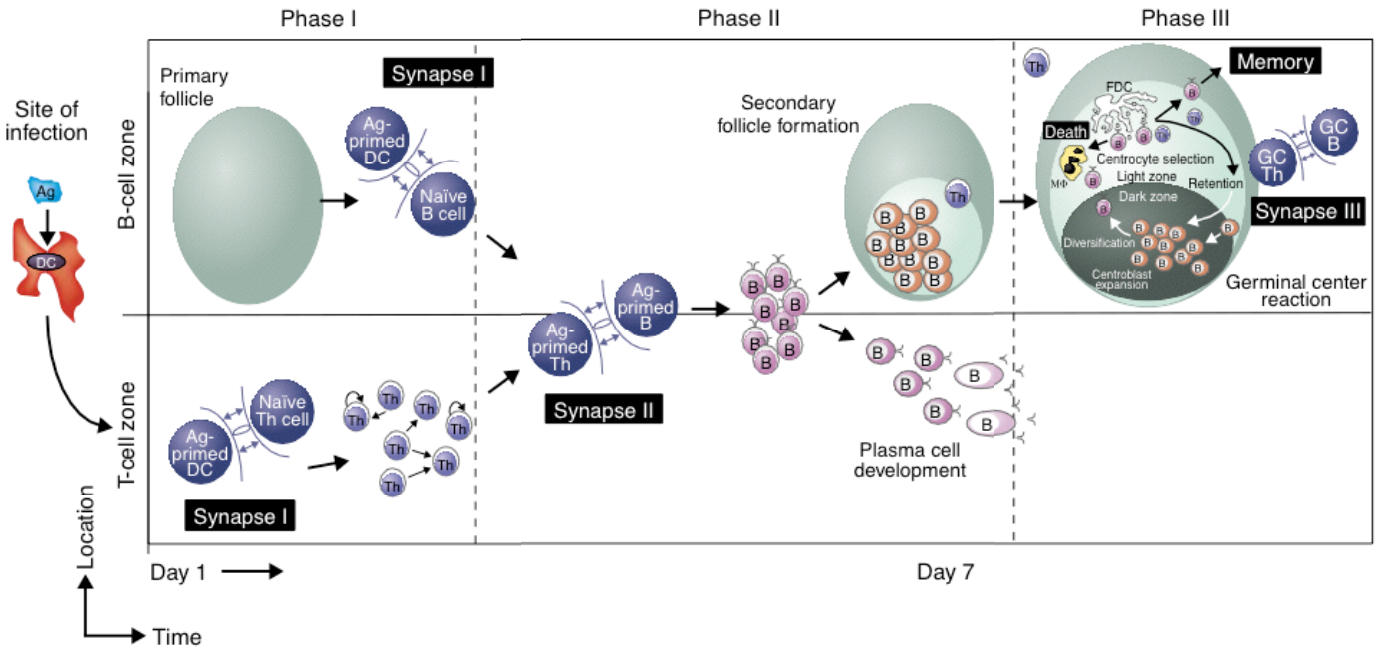


Figure 1.2. Helper T-cell regulated B-cell differentiation. The response begins with migration of activated DC to the LN and triggering of naïve T cell (Synapse I). At the same time B cell get activated either by soluble antigen or antigen-bearing DC. Following clonal expansion, antigen activated T cells contact antigen-specific B cells at the follicular border (Synapse II). At this point B cell undergoes one of two differentiation options, either to become short-lived plasma cell and progress into T cell area or to form secondary follicle in the B-cell zone. The interaction involving germinal center (GC) Th cells and GC B cells (Synapse III) is critical for late stages of B cell development leading to formation of high affinity antibody producing plasma cell and memory cell.
Adapted from M.G. Mc Hayzer-Williams, Current Opinion in Immunology
(McHeyzer-Williams, 2003).

The last phase of TD response, the GC reaction, begins when GC precursor B cells migrate back to a follicle and rapidly proliferate within the environment provided by follicular DCs (FDCs). In spite of their name FDCs are not related to the T zone DCs. They are specialized stromal cells possessing the ability to hold the antigen on their

surface in an unprocessed form for very long periods of time (Park and Choi, 2005; Liu et al., 1996a).
The events occurring in GC (Figure 1.2, Phase III) are described in more detail below.

- Germinal Center reaction

TD B-cell stimulation continues within germinal centers. There, B cells undergo somatic hypermutation with affinity maturation, resulting in secretion of antibodies with an increased affinity for antigen, as well as Ig class switching (Berek et al., 1991; Jacob et al., 1991; Liu et al., 1996b).
During B cell proliferation in the follicle, in the so-called dark zone, the gene segments coding for the antigen-binding site of the antibody are mutated at high frequency, resulting in the generation of diverse progeny (Manser, 2004). This process, called somatic hypermutation, greatly contributes to antibody diversity (Brenner and Milstein, 1966) (Tonegawa, 1983). The rapidly dividing B cells of the dark zone are termed centroblasts and are characterized by low surface BCR. They then exit the cell cycle and migrate to FDC-rich GC area that also contains CD4 T cells, termed the light zone. The light zone B cells, called centrocytes, will die unless are provided with survival signals by FDC that retain antigen on their surface (Rajewsky, 1996; Wagner and Neuberger, 1996). Only the centrocytes, which express mutant BCRs with high affinity for the antigen survive (this process is termed affinity maturation). Later they re-enter centroblast pool or process and present antigen to GC T cells. The latter results in the induction of H chain class switching, and differentiation to antibody secreting plasma cells or to long-lived memory cells (Manser, 2004). The mature naïve B cells express surface Ig of the IgM and IgD class (Pernis and Forni, 1976), the class switch recombination (CSR) however leads to the production of Abs that are better suited to achieve certain biological function(s). During CSR immunoglobulin heavy chain constant gene segment Cμ is replaced by Cγ, Cε and Cα, switching immunoglobulin isotype from IgM to IgG, IgE and IgA, respectively (Honjo et al., 2002). IgM, besides representing the major membrane-bound form of immunoglobulin expressed by mature B cells, is mainly secreted (as a pentamer) in primary immune responses, it activates complement and phagocytic cells. IgG is the most abundant class in serum, it is the major type of Abs secreted during

secondary immune responses. IgA (a dimer or tetramer) is predominant in external secretions such as milk, saliva, tears and gastrointestinal tract mucous. It is a first line of defense at mucous membrane surfaces, which are entry sites for most pathogenic organisms. IgE activates mast cells and basophils via Fc receptors. This class is important for parasite immunity, but also can cause allergies. No biological function has been identified for IgD yet.

1.2. Lymphocyte Activation Gene-3

1.2.1. Identification and the predicted structure

Lymphocyte activation gene - 3 (LAG-3 /CD223) was first identified as a cell surface molecule selectively expressed in activated human NK and T lymphocytes (Baixeras et al., 1992; Triebel et al., 1990). Interestingly, it was found to be related to CD4 at both gene and protein level. The two genes are located next to each other in human and mouse genomes (on chromosome 12 in human and chromosome 6 in mice). Actually, Lag-3 gene is located within the enhancer region of the CD4 gene. Furthermore, CD4 and LAG-3 have a very similar intron/exon organization. It has been, therefore, suggested that they might have evolved from the common ancestor (Huard et al., 1994a). The overall amino acid sequence similarity of LAG-3 with CD4 is barely above the background level (< 20% sequence identity) but there are notable patches of identity with stretches of amino acid sequences and the two proteins probably share structural homology at their four extracellular Ig-like domains (Figure 1.3). However, their cytoplasmic regions clearly have diverged (Huard et al., 1994a; Triebel et al., 1990). The amino acid sequences of intracellular tails of CD4 and LAG-3 are compared in Figure 1.4.

Mouse LAG-3 shows 70% protein sequence identity to its human homologue. All putative structurally important residues are well conserved thus predicting the same folding pattern (Mastrangeli et al., 1996). Also, the RGD motif in D1 domain is conserved in human and mouse. RGD motif can be recognized by integrins and thus modulate cell adhesion (D'Souza et al., 1991). The mouse LAG-3 cDNA and protein sequence, including all the special features of LAG-3 are shown in Figure 1.5.

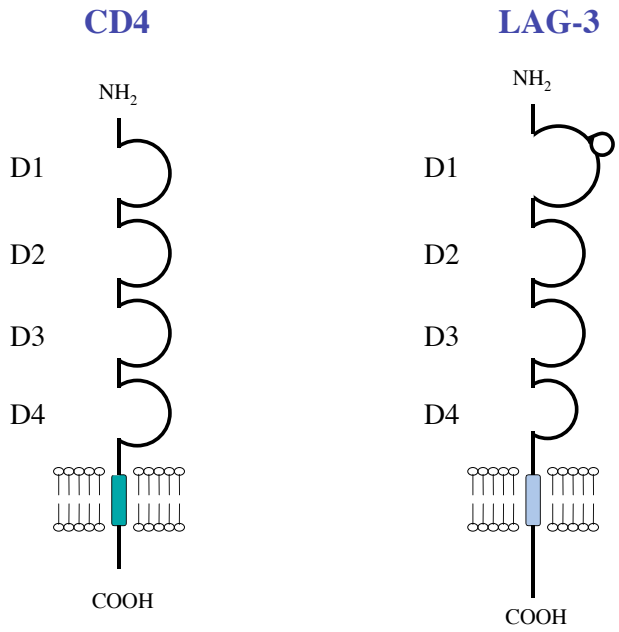


Figure 1.3. The predicted structure of LAG-3 is very similar to the structure of CD4. Both proteins contain four immunoglobulin-like domains, a stretch of hydrophobic transmembrane residues and a cytoplasmic tail. The LAG-3 molecule is slightly bigger with predicted molecular weight of 70 kDa, as compared to 55 kDa of CD4. The amino-terminal domain, D1, of LAG-3 contains a unique looplike insertion.

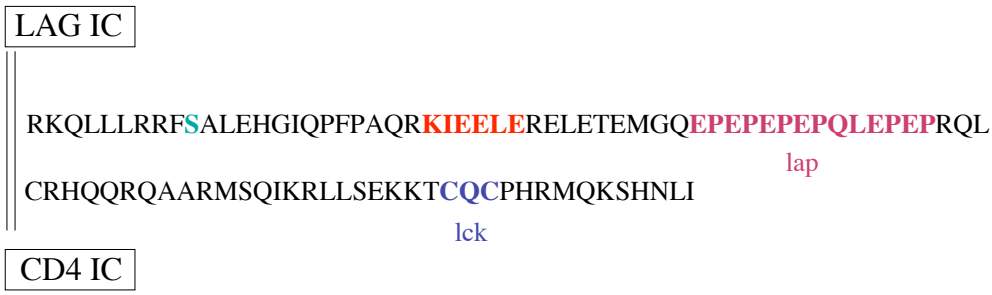


Figure 1.4. The amino acid sequences (one letter code) of the mouse intracellular tails of LAG-3 and CD4. The LAG-3 tail is longer and does not contain the lck binding site. Yet it has three distinguishable motifs: potential serine phosphorylation site (light blue), KIEELE motif (red) and Glutamic acid-Proline (EP) rich region (in pink). All three motifs are conserved between human and mouse (Mastrangeli et al., 1996).

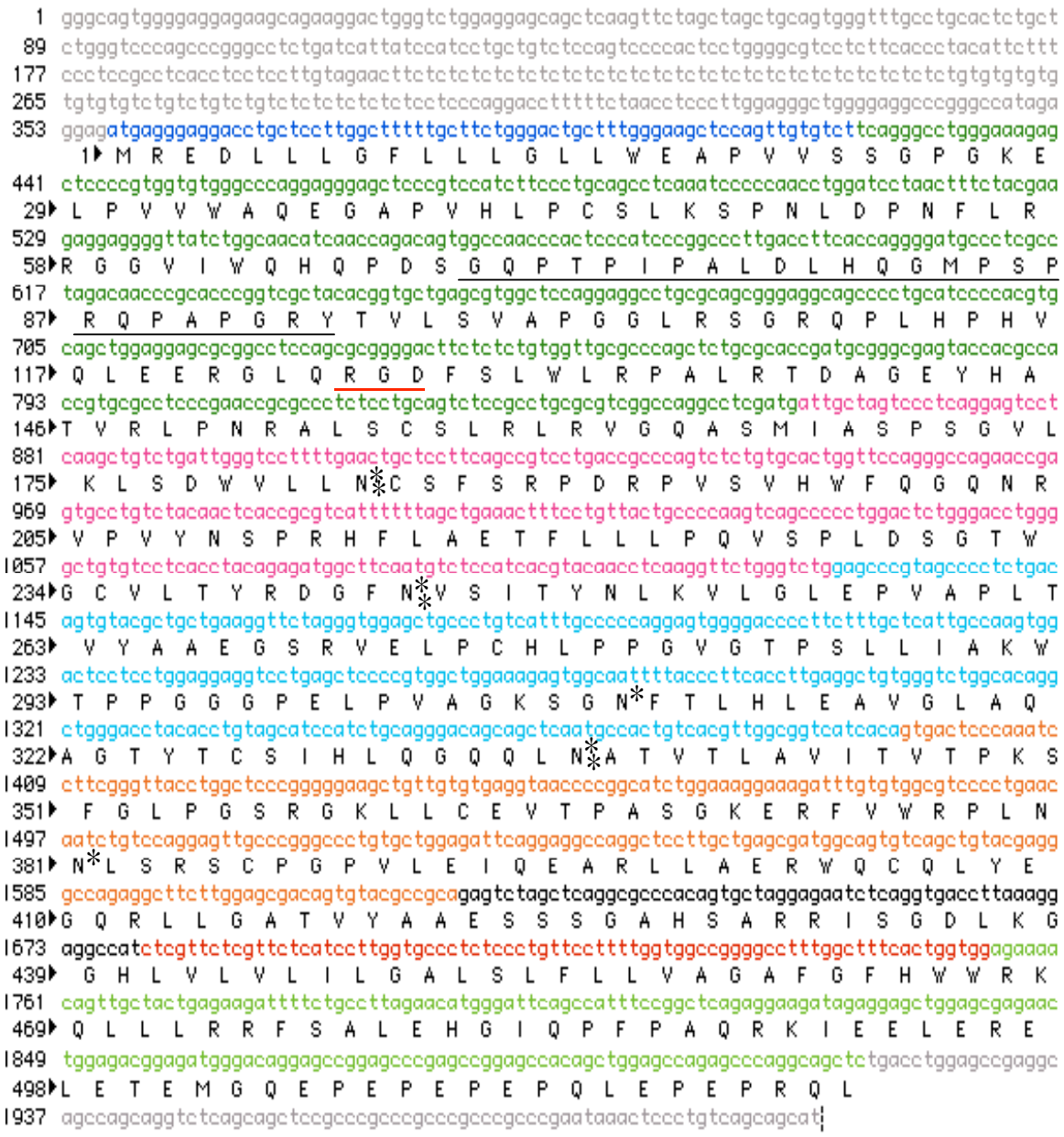


Figure 1. 5. Coding sequence of Lag-3. Coding sequence for leader peptide is indicated in blue, domain 1- dark green, domain 2 – magenta, domain 3 – cyan, domain 4 – orange, connecting peptide –black, transmembrane region – red, intracellular part –light green, non- coding sequence is shown in grey. Extra loop of D1 domain is underlined in black. RGD motif is underlined in red. Potential glycosylation sites overlapping with human counterpart are indicated with double asterisk. Single asterisk marks glycosylation site specific for mouse LAG-3.

Human LAG-3 has four potential N-glycosylation sites, Asn-X-Thr/Ser, where X can be any amino acid residue except for Pro (reviewed by Yan et al. (Yan and Lennarz, 2005)). The immunoprecipitation experiments on human activated T and NK cell lysates showed that 70 kDa LAG-3 specie is indeed glycosylated. Upon N-glycosidase F treatment mobility shift from 70 kDa to 60 kDa was observed (Baixeras et al., 1992). Mouse LAG-3 protein sequence contains five potential N-glycosylation sites, of which 3 (Figure 1.5) overlap with human counterparts (Mastrangeli et al., 1996), but so far there have been no reports that any of them is used.

The close relationship between LAG-3 and CD4 has been strengthened by the demonstration that both share the same ligand, which is MHC class II molecules on the surface of antigen presenting cells (APC) (Baixeras et al., 1992). However, LAG-3 was shown to bind MHC class II with much higher avidity (at least 100 fold) suggesting that it might act as natural competitor of CD4 (Huard et al., 1995; Workman et al., 2002b). This notion was supported by experiments showing that LAG-3 can interfere with MHC class II-CD4 interaction (Hannier et al., 1998; Huard et al., 1996; Huard et al., 1995; Huard et al., 1994b), although LAG-3 and CD4 binding sites on MHC class II do not overlap (Baixeras et al., 1992).

Most of the residues that are essential for interactions with MHC class II proteins are clustered at the base of the extra-loop structure in the LAG-3 D1 domain. The deletion of the loop itself also affects MHC class II adhesion. D2 domain is required for successful LAG-3 – MHC class II interactions as well. It is either involved in the binding itself, or in positioning of D1 for interaction. Domains 3 and 4 were found to be dispensable for binding to MHC class II (Huard et al., 1997).

Interestingly, unlike CD4, LAG-3 does not bind to human immunodeficiency virus (HIV)(Baixeras et al., 1992).

1.2.2. LAG-3 expression pattern

A number of activated human T and NK cell lines, as well as PHA blasts of CD4 and CD8 T cells express LAG-3 on their surface (Annunziato et al., 1997; Annunziato et al.,

1996; Hannier and Triebel, 1999; Scala et al., 1998; Triebel et al., 1990). In immunofluorescence studies, expression of human LAG-3 protein was reported mainly on activated T cells in the inflamed secondary lymphoid organs, with a few scattered positive cells in normal lymphoid organs. The morphology and distribution of LAG-3 + cells were consistent with that of lymphocytes. No LAG3+ cell (Huard et al., 1994a) was found in nonlymphoid organs including muscle, nerve and kidney. The distribution of LAG-3, compared with CD3 and CD25, in normal human tissues is shown in Table 1.1.

	N		CD3	CD25	LAG-3*
Lymph node	9	GC	++	+	+
		T-cell area	+++	+	+
		Sinuses	–	–	+
Tonsil	5	GC	+/++	+	+
		T-cell area	+++	+	+
Spleen	6	White pulp	++	+	0/+
		Red pulp	++	+	0/+
		Sinuses	–	–	+
Thymus	2	Cortex	+++	–	0/+
		Medulla	+++	0/+	0/+
Bone marrow	5		++	0/+	0/+
Malt	2		+	0/+	0/+
Muscle	3		0/+	–	–
Kidney	2		+	0/+	–
Nerve	1		0/+	–	–

Table 1.1. The distribution of LAG-3 in normal tissues, detected by staining of tissue sections with mAb anti-human LAG-3 antibody. GC-germinal centers, Malt- mucosa-associated lymphoid tissue, - no positive cells, + a few positive cells, ++ many positive cells, +++ most cells positive (Huard et al., 1994a).

Consistent with human studies, mouse antigen-activated T cells (CD4 and CD8 populations) as well as poly(I)-poly(C), the synthetic double-stranded ribonucleic acid, activated NK cells express LAG-3 on their surface (Miyazaki et al., 1996; Workman et al., 2002b).

Northern blot analysis of mouse tissues revealed LAG-3 transcripts only in the spleen, thymus and brain and not in stomach, liver, lung, testis, heart, kidney and skeletal muscle. Furthermore, a very small percentage of T and NK cells in naïve mice express surface LAG-3, about 2% and 10 % respectively. Interestingly, in contrast to αβ T cells, significant percentage of γδ T cells (almost 19 %) show LAG-3 expression (Workman et

al., 2002b). In addition, functional genomic screen of TCRγδ intraepithelial lymphocytes revealed LAG-3 mRNA (Fahrer et al., 2001).

However, it must be mentioned that all the above in vivo T cell expression data is reported in the form of tables and no primary results are shown (Huard et al., 1994a; Workman et al., 2002b).

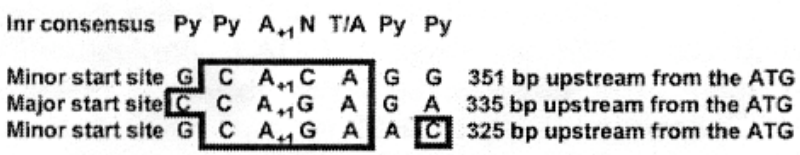
1.2.3. Regulation of Lag-3 expression

The studies concentrating on potential factors inducing LAG-3 expression on human T and NK cells showed that certain cytokines: IL-2, IL-7, IL-12 up-regulated surface LAG-3 expression, while others: IL-4, IL-6, IL-10, TNF-α, TNF-β and IFN-γ did not (Bruniquel et al., 1998). In fact, IL-12 proved to be the most potent stimulus for LAG-3 expression on activated T and NK cells (Annunziato et al., 1997; Bruniquel et al., 1998). None of the above mentioned cytokines were able to down-regulate LAG-3 expression.

Aditionally, the most activated CD4+ T cell clones with established Th1 profile of cytokine secretion expressed LAG-3 on their surface, whereas the great majority of Th2 clones showed neither surface LAG-3 nor LAG-3 m RNA expression (Annunziato et al., 1996; Scala et al., 1998).

The analysis of the 5' region of human Lag-3 gene revealed that LAG-3 has a TATA-less promoter and, like many genes lacking a TATA box, it has multiple transcriptional start sites. The LAG-3 transcription initiation sites are the putative Inr motives, as shown in Figure 1.6a. Inr sequence is another transcriptional initiator DNA element (Javahery et al., 1994). In addition, a number of regulatory regions upstream from the transcription start site have been identified including the DNA binding motifs of GATA, c-Ets, NF-kB and NFAT transcription factors and GC box, the putative binding site of Sp1 transcription factor (Figure 1.6b) (Bruniquel et al., 1998). In particular, -1061/-541 region was identified as positive regulatory element, while +211/+311 located in the first exon was found to hamper the activity of regulatory elements upstream of the transcriptional start site.

a)



b)

```
-1371   TATGTCCTGT TTTGGATTCT CAGGGATGCT CCTCCCCAGC AATACACACT
-1321   TCCCTGCTAA CCCACACAAG ACCTAGACAC TGCTGAGCCC AGTGGAAAGC
                                          GATA-1          c-Ets-1
-1271   TACCAGCTCT ATTGTGCTGA ACCAGGCACA GAGAAACAGC CCAGAAACAG
-1221   GAAGTCCTGC CCCTGAGCTG GGAGAGGGCT GGTAGTCCTG GGTCTCAGCC
-1171   TGCTAGGATC TGGAGTGAGC TGCCTGGCAT CAGAAATGCC CTCTGCCTGG
-1121   CATGGAAGCG AGCTCCTTCC AGTCAAGCAG GGAAATCCTG GGTCTCGTGT

-1071   CATCCCCAGG TCGGCTGTAT GTTTCGTCAC CTGCCCTCCA ACTCTCCCTG
                                                    NF-KappaB
-1021   GGCTGGGCTG GGGAGCGGGA GTTGTATGAC TCTTGTCAGT CTTGGGCCTT
-971    CCCTAGTCCC ACTGCCTCTG TTTCCCCTGA GTTTGATCTC TGTTAGGAGA
                                                      NFAT-1
-921    TTTCTAAGAT TTCTGCATTT CTCCATCTCC GCATGGCCCT TGCCTTCTCC
-871    TCTTAGTTCG GAACCTCTGG ACTGGAGGAT GCTTCTGAGG GCATGGGGAC
-821    CTGAGTTGGA GTGGGGAGGG GTGTTGACTC ATCAACAGCA TCATTTCTCC
-771    CAGGAGACGC CCGTAACTCA TCCAAGGTCA GGACTACACA CTGTCACGCA
-721    CACAGGTACA CTCACAGCTT TTATCTTCAC GCTCCCTAAC CTTGGCAAAT
-671    TGTACAATTT CTTTGAAGCT CAGTTTCCTC ATCTGTACAA TGGGGAAAAG
-621    CATTAGATTT CATGAATTAC TATAAGTAAA GTGTCCAATA CAGTGCTTAG
-571    CACGTAATGA AGCCTCAATA CAATGTTAGT TATTCTCCAT GCCCCACAAA
-521    GCTGGCATGC CTAGCCTCAG ACCTACCATT TTTTGGGGTG CAGTAAGGCT
-471    TCCTGTCCAC CATGTTCCCA GGGACATTGT ACTGATGGGT GGAAAGGCAG
-421    GTCTAAAGGG GTCACGAAGT TCTGGGAGGT TAAGGGAACG AGGAAGGAGA

-371    TTGAGCAACA AGGAAAGAGC TTGCCAAGAA GGAGGTGTGA ATATTGGGAC
-321    TGAGGAGGCA GCTTAGAGAT GGGCAAGGGG GCAGTTCCAG GCAGAAATGG
-271    TTCGTGGAGG CAGAAGGTCC CTGGGAGAGG GAGCAGTCTG GAGGGTGGGG
-221    CAGGGGCGAG GAGGGGGAGG TGGGGAGACC CAGGACTGAG GAAGTAAACA
-171    AGGGGAGCGC CACCACAGAG GTGGAGAGGT GGAGGGTGCT GCTGCTGGGA
-121    ATCAACCCCC TCAGACTTTC CACTGCGAAG CGAAACCGTA AGCCCTGGGG
                GC Box
-71     TGCGGGGGGC GGGCCGGGAG GAGGGGAAGT GGGGAAGGTG GAGGGAAGGC
          (+1)                +1          (+1)
-21     CGGGCACAGG GGTGAAGGCC CAGAGACCAG CAGAACGGCA TCCCAGCCAC
+30     GACGGCCACT TTGCTCTGTC TGCTGTCCGC CACGGCCCTG CTCTGTTCCC
+80     TGGGACACCC CCGCCCCCAC CTCCTCAGGC TGCCTGATCT GCCCAGCTTT
+130    CCAGCTTTCC TCTGGATTCC GGCCTCTGGT CATCCCTCCC CACCCTCTCT
```

Figure 1.6. The human LAG-3 5' region. a) multiple Lag-3 transcription initiation sites are compared with Inr sequence. b) The major transcription start site is marked as +1 and the minor sites as (+1). The potential transcription factor binding sites are indicated. Adapted from Bruniquel et al. 1998.

1.2.4. Suggested function of LAG-3

The physiological role of LAG-3 is not clear. Since LAG-3, like CTLA-4, is expressed only after lymphocyte activation, it cannot play a role in the induction phase of the response. Because it has a higher affinity for MHC class II than CD4 (and therefore potential for displacing CD4), however, it was thought that LAG-3 might act as a negative regulator of T cell activation. Consistent with this prediction, addition of blocking anti-LAG-3 antibodies to IL-2 dependent human T cell lines resulted in sustained proliferation, increased CD25 activation marker expression and increased INF-γ and IL-4 secretion (Huard et al., 1994b). Subsequent studies with polyclonal human T cells showed that TCR-induced activation was inhibited by anti-LAG-3 Ab cross-linking (Hannier et al., 1998).

It was also demonstrated that LAG-3 co-caps with CD3 at the immunological synapse, but surprisingly it was preferentially associated with the CD8 rather than the CD4 co-receptor at the cell surface (Hannier and Triebel, 1999). In addition, LAG-3 was found to be enriched in lipid rafts of activated T cells (Iouzalen et al., 2001). Lipid rafts are detergent insoluble, cholesterol and glycosphingolipids rich membrane microdomains, which have been implicated in signal transduction, cholesterol trafficking and endocytosis (Pike , 2004).

The initial analysis of LAG 3-/- mice did not reveal any defect in T nor B cell function (Miyazaki et al., 1996). But two more recent studies showed that murine LAG-3 can act as regulator of T cell expansion. These studies, however, showed different effects *in vitro* than *in vivo*. *In vitro,* defective expansion of activated LAG3-/- T cells after staphylococcal enterotoxin B (SEB) stimulation was observed. The effect was attributed to the increased cell death, since the cells were proliferating at the normal rate (Workman and Vignali, 2003). Similar observations were made with LAG3-/-CD4+ OT-II transgenic T cells following antigenic peptide stimulation. Antigen-driven expansion was restored by constitutive expression of LAG-3 via retroviral-mediated stem cell gene transfer (Workman and Vignali, 2003). Yet, *in vivo*, LAG3-/- T cells exhibited a delay in cell cycle arrest following stimulation with SEB resulting in increased T cell expansion and splenomegaly. Moreover, increased T cell expansion was observed in adoptive recipients of LAG-3 -/- OT-II transgenic T cells following *in vivo* Ag stimulation

(Workman et al., 2004). Similarly, the infection of LAG3-/- mice with Sendai virus resulted in increased numbers of memory and CD4+ and CD8+ cells (Workman et al., 2004). This *in vivo* data supports the idea of LAG-3 being a negative regulator of T cell activation and expansion.

The initial analysis of LAG3-/- deficient mice, nevertheless, showed a defect in the natural killer cell compartment. Killing of certain tumor targets by NK cells from these mice was inhibited or even abolished, whereas lysis of cells displaying MHC class I disparities remained intact. It was thus suggested that LAG-3 is a receptor or co-receptor defining different modes of NK killing (Miyazaki et al., 1996). This notion, however, was not supported by studies with human NK cells (Huard et al., 1998).

Very recently, a new aspect of LAG-3 function was investigated. It was suggested that surface LAG-3, besides modulating effector T cell activity, influences regulatory T cell suppressor activity. Not only CD4+ CD25+ T cells from LAG3-/- mice exhibited reduced regulatory activity, but also ectopic expression of LAG-3 on CD4+ T cells conferred on them suppressor activity toward effector T cells (Huang et al., 2004).

LAG-3 expression is thought to be limited to activated T and NK cell populations, but it has been shown to exert an effect on other cell types as well, particularly monocytes and dendritic cells. For example, LAG-3 expressed on the surface of human proinflammatory bystander T cells (that is T cells in co-cultures with autologous monocytes and IL-2, activated in the absence of TCR engagement) synergizes with low level of CD40 L expressed on these cells to trigger TNF-α and IL-12 production by monocytes (Avice et al., 1999). Furthermore, in the same way as soluble CD40 L, soluble LAG-3 can directly induce DCs to produce Th1- cytokines and chemokines, known to direct the migration of maturing DCs to lymph nodes (Andreae et al., 2002; Buisson and Triebel, 2003).

1.2.5. Mode of action of LAG-3

The mechanism of LAG-3 action has not been clarified. In some studies, it was concluded the extracellular part is important, others suggested that the intracellular tail

and thus the potential downstream signalling pathways play a role. For example, the inhibitory effect of LAG-3 on CD4-dependent T cell function was lost upon deletion of the LAG-3 cytoplasmic tail (Workman et al., 2002a), in accordance with the idea that LAG-3 does not disrupt CD4 - MHC class II interactions but rather directly inhibits T cell activation through intracellular signalling. LAG-3 could, therefore, be considered as an independent (negative) co-receptor, like the other two TCR-assisting MHC ligands, CD4 and CD8. The signal transduction pathways downstream of LAG-3, however, have not yet been delineated. It is clear that the intracellular tail of LAG-3 does not contain any immunoreceptor tyrosine-based inhibitory motif (ITIM) consensus sequences that are usually found in various inhibitory receptors (Burshtyn et al., 1996; Vely and Vivier, 1997). But, three regions of putative importance are conserved in mouse and human (Figure 1.4); EP (Glutamic acid - Proline) repeated motif is a binding site for LAP (LAG-3 associated protein) a candidate molecule for a new type of signal transduction and/or coupling of the immunological synapse to the microtubule networks (Iouzalen et al., 2001; Triebel, 2003). KIEELE motif was reported to be important for the inhibition of CD4-dependent, but not CD4-independent T cell function by LAG-3. While serine 454 is a potential phosphorylation site for casein kinase II, cAMP and cGMP dependent protein kinases (Workman et al., 2002a; Workman and Vignali, 2003). In contast to CD4, the intacellular tail of LAG-3 contains no binding motif for Lck, a protein tyrosine kinase crucial for early signal transduction events downstream of TCR.

Several alternate splice variants of human Lag-3 were described. Two of them encoding potential truncated forms: LAG-3V1 (domains D1 to D2 of 36 kDa) and LAG3V3 (domains D1 to D3 of 52 kDa) suggesting the existence of soluble form of LAG-3 (sLAG-3) (Triebel, 2002). However, very recently it was shown that sLAG-3 isolated from activated mouse T cell hybridoma cultures is a result of proteolytic processing occurring at the connecting peptide region of surface LAG-3 rather than an alterative splicing product. This soluble LAG-3 migrated at apparent molecular weight of 54 kDa. In addition, the cross-linking experiments indicated that LAG-3 is present at the cells' surface as a dimer and in oligomeric form (Li et al., 2004). Thus, it is possible that soluble LAG-3 exists also as a multimer.

The extracellular part of LAG-3 is thought to mediate its effect through binding to MHC class II. It is conceivable that membrane bound LAG-3 and soluble LAG-3 (sLAG-3) perform different functions in the immune system.

The longer form of LAG-3 was detected by ELISA in the serum of healthy individuals as well as tuberculosis patients with favorable outcome (Lienhardt et al., 2002; Triebel, 2003). In fact, since LAG-expression correlates with IFN-γ production by activated T cells, soluble LAG-3 has been even suggested as a serological marker of Th1 responses (Annunziato et al., 1996). Soluble LAG-3 was also detectable by ELISA in the serum of wild type mice (Li et al., 2004). The potential function of LAG-3 has been probed with recombinant soluble LAG-3 composed of extracellular part of LAG-3 fused to human IgG1 Fc region (mLAG-3Ig). Soluble mLAG-3Ig fusion protein induced MHC class II mediated signaling in monocytes and dendritic cells leading to their activation and/or maturation as well as production of cytokines (Andreae et al., 2003; Andreae et al., 2002; Avice et al., 1999; Demeure et al., 2001). Moreover, sLAG-3 stimulated immature DCs had a 4- to 5-fold increased capacity to stimulate naïve T cell proliferation (Demeure et al., 2001). Consistently, co-administration of soluble mLAG-3Ig was shown to increase CTL and CD4 Th1 responses in mice (El Mir and Triebel, 2000).

1.2.6. LAG-3 and cancer

LAG-3 has also been implied to have anti-tumor activity. For example, Lag-3 transfected tumors completely regressed or their growth was markedly reduced in mice, and the challenged mice were significantly protected against a re-challenge with parental tumor cells. Likewise, the co-administration of soluble recombinant LAG-3 (mLAG-3Ig) with wild-type tumor cells also reduced primary tumor growth (Prigent et al., 1999).

Another study, exploiting mammary tumor model in transgenic mice, found that co-injection of soluble LAG-3 (mLAG-3Ig) enabled DNA vaccination with plasmids coding for the oncogene product to establish effective protection against mammary carcinogenesis. It elicited stronger and sustained (unlike DNA vaccination alone) protection and kept 70% of 1-year old mice tumor free (Cappello et al., 2003).

In human, T lymphocytes infiltrating various tumor types (melanoma, renal cell carcinoma, diffuse large cell lymphoma, peripheral T cell lymphoma) were found to express LAG-3 (Demeure et al., 2001).

2. THESIS OBJECTIVES

Properly functioning immune system is essential for our health. Inadequate responses to pathogens or tumors as well as hyper-reactivity leading to destructive autoimmune disease have a direct impact on our survival. Hence, the immune responses have to be under tight control. Especially, the regulation of T cells, the key players of adaptive immunity, their activation and expansion, is crucial for the maintenance of the harmony in the system.

Recently a novel cell surface molecule, called LAG-3 (Lymphocyte activation gene –3 / CD223), was identified which has a potential regulatory role in the immune system. This protein was detectable on activated T cells in human inflamed lymphoid tissues but not in normal lymphoid organs. *In vitro* studies strongly suggested that LAG-3 plays a negative role in T cell activation. However, the physiological role of LAG-3 remains unclear.

The goal of this thesis was to characterize mouse LAG-3. Mouse system allows for more easily accessible and manipulable experimental approach needed to gain deeper insight into the functional significance of this molecule. As there were no reagents available, the first objective was to generate tools enabling the characterization of LAG-3 in the form of (i) monoclonal antibodies against LAG-3, (ii) the recombinant soluble LAG-3, as well as (iii) LAG-3 transduced cell lines. There was only scarce information about murine LAG-3 expression pattern, this surface glycoprotein was found only in activated T and NK cells. Monoclonal antibodies would offer the possibility to undertake detailed analysis of LAG-3 expression pattern and function in the immune system. It would also allow for studying how different activation regimes of purified immune cell populations or their mixtures induce LAG-3 expression. Recombinant soluble LAG-3, on the other hand, would open the possibility to undertake structural investigations of the protein. Therefore, another goal was to find and optimize the conditions for a large-scale production of soluble recombinant LAG-3, in *Drosophila* cells expression system. The purified soluble LAG-3 would be subjected to crystallization trials alone or in complex with its reported ligand - MHC class II. If successful, the detailed structural information may help understanding how LAG-3 interacts with its ligand. LAG-transduced cell lines could give us indications about the biological role of LAG-3.

The last objective was to use LAG-3 deficient mice to confirm the specificity of our reagents and to verify our *in vitro* observations.

All these approaches were aimed at gaining a deeper understanding of LAG-3 function in the immune system.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plastic ware

Flasks, plates, pipettes, roller bottles, conical tubes	Costar/Falcon,
Freezing vials	Nunc Inc.
Microfuge tubes/ PCR tubes	Eppendorf
Filter bottles	Millipore/Corning
Immulon 2 plates	Dynex
FACS tubes	Micronic

3.1.2. Chemicals and additives

Agarose (ultra pure)	Gibco BRL
Aluminium Potassium Sulfate (APS)	Sigma
Ammonium Persulfate (APS)	Amresco
Ammonium sulfate	Fluka
Ampicillin	Sigma
ABTS(2,2'-Azino-bis(3-ethylbenzothiazoline -6-sulfonic acid) diammonium salt	Sigma
β-mercaptoethanol	Sigma
Bacto agar	Difco
Bacto-tryptone	Difco
Bacto-yeast extract	Difco
Bovine Serum Albumin (BSA)	Sigma/Biolabs
Ciproxin (0.2 g in 100ml solution)	Bayer
CFSE (carboxyfluorescein diacetate, succinimidyl ester)	Molecular Probes
Copper sulfate ($CuSO_4$)	Fluka
Dextran sulfate (sodium salt)	Pharmacia
DEPC (diethyl pyrocarbonate)	Sigma
Dithiothreitol (DTT)	Sigma
DMSO (dimethyl sulfoxide)	Fluka
EDTA (ethylenediaminetetraacetic acid)	Fluka
Ethanol	Merck
Ethidium bromide	Merck
FCS (Fetal Calf Serum)	Seromed
Glycerol	Sigma
Glycine	Fluka
HeaIII/HindIII DNA size marker	New England Biolabs (NEB)
HAP	Bio-Rad
HAT(50x)	Boehringer/Roche
HCL	Merck
HEPES	Sigma

H_2O_2	Fluka
HT (50x)	Boehringer/Roche
Hygromycin	Calbiochem
IMDM powder stock	Gibco
Insulin	Sigma
Isopropanol	Merck
Leupeptin	Sigma
LPS (lipopolysaccharide)	Sigma
MEM non essential amino acids 100x solution	Gibco BRL
Methanol	Merck
Milk (powder, blotting grade)	Bio Rad
NaOH (10M)	Fluka
Nonidet P-40 (NP-40)	Fluka
Pepstatin A	Sigma
Paraformaldehyde (PFA)	Sigma
PEG (polyethylene glycol) 1500	Boehringer
Penicillin /Streptomycin 100x solution	Gibco BRL
PMSF (phenylmethylsulfonyl fluoride)	Sigma
PRIMATONE ® RL/LF	Quest International
Puromycin	Calbiochem
Sodium azide	Fluka
Sodium dodecyl sulfate (SDS)	Bio Rad
Sodium carbonate (NaHCO3)	Fluka
TEMED (N,N,N',N' - tetramethylethylene diamine)	Amresco
Tris	Merck
TNF-α	Pharmingen
Triton X-100	Bio Rad
Tunicamycin	Sigma

Molecular biology enzymes were purchased from BioLabs, Roche or Gibco BRL.

3.1.3. Media and Buffers

IMDM medium	IMDM powder for 10l 30.24 g $NaHCO_3$ up to 10 l Millipore H_20
Serum free-medium:(SF-IMDM)	IMDM containing: 50μM β-mercaptoethanol 50 units/ml Penicillin 50 μg/ml Streptomycin 1 x non-essential amino acids 5 μg/ml Insulin 0.03 % Primatone RL

LB medium:	10g Bacto-tryptone 5g Bacto-yeast extract 10g NaCl H_2O up to 1l
LB agar:	LB medium 1l 15g Bacto agar
SOB-Mg	10g Bacto-yeast extract 0.5g NaCl 10ml 250mM KCl H_2O add 1l
SOB – Mg agar plates	SOB- Mg medium 1l 15g Bacto agar
EWB	10% redistilled glycerol in ultrapure water
TE	10mM Tris-HCl (pH 8.0) 1mM EDTA
Phosphate buffered saline (PBS):	80g NaCl 28.8g $Na_2HPO_4x12H_20$ 2g KCl 2g KH_2PO_4 add up to 10l H_20 bidestilled
1xGey's solution:	20 parts stock A 5 parts stock B 5 parts stock C 70 parts cell culture grade H_20
Stock A:	NH4Cl 35.0g KCl 1.85g Na2HPO4.2H2O 1.5g (or Na2HPO4.12H2O 0.75g) KH2PO4 0.12g Glucose 5.0g Phenol red 50.0mg in 1000ml H_20
Stock B:	MgCl2.6H2O 0.42g MgSO4.7H2O 0.14g CaCl2 0.34g (or CaCl2.2H2O 0.45g) in 100 ml H_20)

Stock C:	NaHCO3 2.25g in 100ml H_20
Rnase-free water	DEPC-treated water
SF-900 II SFM	Gibco
DMEM	Gibco
HBSS without Ca2+ and Mg2+	Sigma
TAE	40mM Tris/acetate, pH 8 1mM EDTA
Tris-glycine electrophoresis buffer	25mM Tris 250 mM Glycine (pH 8.3) 0.1% SDS
SDS-PAGE sample buffer 4x	200 mM Tris-CL (pH 6.8) 8% SDS 0.4% bromophenol blue 40% glycerol
Glycine - HCL buffer pH 3.2	50ml of 0.2M glycine 8.2ml of 0.2M HCL 141.8 ml H_20
Glycine - HCL buffer pH 2.8	50ml of 0.2M glycine 16.8ml of 0.2M HCL 133.2 ml H_20
ABTS substrate solution	for 20 ml 18ml Mc Ilvain's buffer 2 ml ABTS (10mg/mL) 1% H2O2
OptiprepTM dilution buffer	0.88% NaCL 1mM EDTA 10mM Hepes-NaCl 0.5 % BSA pH 7.4 filter sterilized 0.2 μm
IL-2, IL-4, Il-6	X63 cell line carrying expression vector for a given cytokine (Karasuyama and Melchers, 1988)

3.1.4. Special Reagents

CpG 1826	Microsynth, Balgach, Switzerland
OVA 323-339 peptide	Seccion de Proteomica, Universitat Pompeu Fabra, Barcelona, Spain
HA 110-119 peptide	Basel Institute for Immunology peptide facility, Basel
dNTPs	Amersham Pharmacia
T4 ligase	Roche
Reverse transcriptase	Roche
Ficoll-PaqueTM	Pharmacia
OptiprepTM	Nycomed
DMRIE-C Reagent ®	Gibco BRL
Lipofectin®	Gibco BRL
Lipofectamine ®	Gibco BRL
GELCODE® Blue Stain Reagent	Pierce
BENCHMARK^{TM} protein size marker	Invitrogen
Collagenase D	Boehringer Manheim
TRIzol	Gibco
Hyflo Super Cell ®	Fluka

Fluorescently labelled antibodies were purchased from BD Pharmingen.

Unlabeled antibodies were produced and purified from respective hybridomas. When needed purified Abs were biotinylated.

Rabbit polyclonal anti-mouse LAG-3 Ab	was produced in our lab by Giuseppina Sollami
Goat anti-rabbit horseradish peroxidase HRP) liked Ab	Southern Biotechnology Associates, Inc.
Goat anti-rat Ig HRP linked conjugate	Tagoimmunologicals
Goat anti-rat IgG-biotinylated	Pharmingen
Goat anti-rat IgG2a-biotinylated	Pharmingen
Goat anti-rat IgG2b-biotinylated	Pharmingen
Streptavidin - Texas Red	Molecular Probes

3.1.5. Other materials

Electropration cuvette	Bio Rad
MACS LS Separation columns	Miltenyi Biotec
Streptavidin MicroBeads	Miltenyi Biotec
PD-10 Columns	Amersham
CNBr activated Sepharose	Pharmacia
G+ Sepharose	Pharmacia
Poly-D lysine	Sigma

Nylon Net (Nitex)	Nytal
Microscpe Slides, SuperFrost Plus	Menzel-Glaser
Cover Slips, D=15 “Assistant”	Ominilab

3.1.6. Kits

Geneclean TM	Bio 101
Plasmid mini-prep GenecleanTM RPM	Bio 101
Plasmid maxi-prep	QIAGEN
DNA Ligation Kit	Roche
Gel purification kit GenecleanTM	Bio 101
BigDye® Terminator Cycle Sequencing Kit	Applied Biosystems
ECL Kit	Pharmacia

3.2. Methods

3.2.1 Molecular biology techniques

- preparation of electro-competent bacteria

Bacteria used for transformations were prepared in the following way: single colony of E.coli (strain DH10B or XL-Blue) was picked form freshly streaked agar plate and resuspended in 1mL of SOB-Mg medium by vortexing. The cells were used to seed 50 mL of SOB-Mg medium and grown overnight at 37°C and 275 rpm. The following day 7.5 mL of this fresh culture were used to inoculate 750 ml of SOB- Mg medium. The bacteria were incubated at 37°C with moderate agitation until optical density of 0.75 units at 550nm was reached (corresponding to 3-6 x 10^8 cells per ml). Then cells were collected by centrifugation at 2600g for 15 min. at 4°C. The bacteria were resuspended in 750 ml of EWB, centrifuged, and the wash step was repeated. The pellet was resuspended in the few drops of liquid left in the centrifugation tube. Volume was then adjusted with EWB to obtain OD 550 >200 units and frozen in 50 μl aliquots and stored at –70°C.

- transformation of bacteria

An aliquot of electro-competent bacteria was thawed on ice, 1 μl of DNA solution (plasmid preparation or ligation mix, ethanol and detergent free) in 0.5 TE was added.

The cells were kept 1 min on ice, transferred to pre-chilled electoporation cuvette and electroshocked (12.5 kVper cm, 25 μF, 200 Ohms). Immediately after shock, 1 mL LB medium was added to the cuvette and the cells were transferred into an Eppendorf tube and incubated at 37°C for 1hr, with shaking. The bacteria were plated on agar plates containing the selection antibiotic ampicillin (50 μg/ml) and incubated o/n at 37°C.

- ligation of DNA fragments into a plasmid vector

Ligations were performed in total of 10 μl volume using 1 Weiss units of T4 ligase in the buffer supplied with ligase by the manufacturer for 1hr at room temperature. 1:3 vector to insert ratio was used. Vectors and inserts digested with appropriate restriction enzymes were purified using Geneclean TM purification kit.

- restriction digests and analysis of plasmid DNA

In order to analyse plasmid DNA or prepare fragments for ligation, the DNA was digested with the appropriate restriction enzyme in the buffer supplied with the enzyme. The cut DNA was analyzed on 0.8 to 2% (depending on the size of the fragment) agarose gel containing ethidium bromide (allowing visualization of DNA under UV light). The gels were run in TAE buffer at 80 V.

- small and large scale plasmid preparation

For small-scale plasmid preparation a single bacterial colony was transferred into 2 mL of LB medium (ampicilin 50 μg/ml) and grown o/n at 37°C with vigorous shaking. 1.5ml of the culture was transferred to a microfuge tube and cells were harvested by centrifugation. The Geneclean ^{TM} RPM Kit was used according to the supplied protocol.
For large plasmid DNA preparation bacteria from 100 ml overnight culture (seeded with 2 mL of small scale culture) were harvested by centrifugation at 6000 g at 4°C for 15 min and the QIAGEN® maxi plasmid purification protocol was followed using supplied reagents.

- DNA sequencing

For sequencing of DNA constructs, 2 µl of plasmid DNA (200-500 ng) was mixed with 2 µl of appropriate primer (10 µM), 8µl of H_20 and 8µl of BigDye sequencing mix (Applied Biosystems). The reaction was subjected to 25 extension cycles (96°C 30 sec., 50°C 15 sec., 60°C 2 min.) and followed by isopropanol precipitation (2 µl of 3M NaAc and 20µl of isopropanol). The pellet was dissolved in 18 µl of TCR (template suppressor reagent, BigDye® Terminator Cycle Sequencing Kit), heated up to 95 °C for 5 min. and run on Genetic Analyzer 310 (Applied Biosystems).

- RNA purification

RNA was purified using the TRIazol reagent. Cells were lysed by pipetting up and down in the appropriate amount of TRIazol reagent (1ml per 5-10x10^6 cells). The mixture was additionally incubated for 5 min. at RT, then 0.2 ml of chloroform was added per 1 ml of TRIazol used at the beginning, the tube was shaken vigorously by hand for 15 seconds and incubated for 3 min. at RT. Following centrifugation at 12000x g for 15min. at 4°C, the upper (aqueous) phase was transferred to a fresh tube and RNA was precipitated by the addition of 0.5 ml of isopropanol per 1ml TRIazol used for cell lysis. After another centrifugation and washing with 70% ethanol, RNA was dissolved in RNase-free water, and the A_{260} was measured to determine the yield.

- RT-PCR reverse transcription - polymerase chain reaction

For the first strand synthesis the RNA was dissolved in 10µl RNase-free water, 500ng of the cDNA synthesis oligonucleotides (random hexamers) was added and the mixture was heated for 15min. at 65ºC. Then the following components were added:
6.6µl 5x reverse transcriptase buffer
1 µl 0.1M DTT
3.3µl 10mM dNTP's
1.3µl BSA 2.5mg/ml
1µl human placental RNase inhibitor

1.5 μl Superscript RNase H-MMLV reverse transcriptase
RNase-free water up to 33 μl was added.
In that volume up to 5μg mRNA can be reverse transcribed.

The obtained cDNA was used as a template for PCR. PCR is a technique that allows the amplification of a given DNA fragment that is flanked by short known sequences. The DNA from which the DNA fragment has to be amplified, is mixed with large excess of single-stranded oligonucleotides that are complementary to the flanking sequences, Taq polymerase, dNTP's and the buffer. One oligonucleotide is complementary to the antisense of the 5'-flanking sequence, the other to the sense of the 3'-flanking sequence and they are typically 20 bp long. When the mixture is heated the DNA gets denatured, then the oligonucleotides can anneal to the DNA and the Taq polymerase can amplify the appropriate fragment. The program used for LAG-3 amplification from cDNA was:

Step 1: 2' 95°C
Step 2: 20" 95°C
Step 3: 10" 55°C,
Step 4: 90" 72°C
Step 5: step 2 to 4 - 29 times
Step 6: 10' 72°C

The products of the reaction were visualized on a 1.5% agarose gel.

- preparation of expression construct for production of soluble LAG-3

The coding sequences for D4t (nucleotides 357-1769) and D2t (nucleotides 357-1122) of Lag-3 were amplified from thymus cDNA by PCR using primers with EcoRI restriction site overhang (forward primer) and BglII restriction site overhang (reverse primer) and cloned into pRmHa-3 vector multiple cloning site by standard molecular techniques. The result is shown in Figure 3.2.1. The mini-preparations of the potential plasmid clones were sequenced to make sure that no mutations were introduced during PCR reactions. Subsequently maxi-preparations of pRmHaLAG-3D4t and pRmHaLAG-3D2t were done.

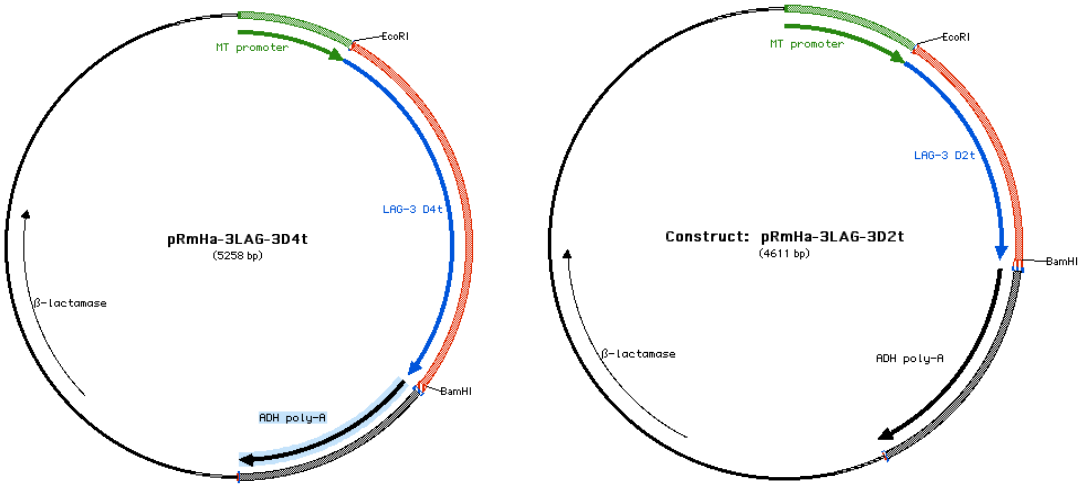


Figure 3.2.1 The DNA constructs for LAG-3 truncations: D4t (domains D1 to D4) or D2t (domains D1 to D2) were cloned into pRmHa-3 *Drosophila* expression vector, under the control of *Drosophila* metallothonein (MT) promoter and in front of *Drosophila* alcohol dehydrogenase polyadenylation signal (Bunch et al., 1988).

3.2.2. Biochemical techniques

- purification of monoclonal Abs

A monoclonal antibody-producing hybridoma cell line was grown in 2% FCS SF-IMDM in roller bottles until 20-30% of cells were dead. The culture supernatant was filtered over Hyflo Super Cell ® on 0.45um filter to remove cells and prevent the clogging of the subsequently used affinity column. Sodium azide (0.05% final) was added to the supernatant and the supernatant was run twice through protein G+ Sepharose column at 4ºC. After washing with 10-20 column volumes of PBS the antibody was eluted in 3ml fractions with HCL-glycine buffer of pH 2.8. Elution was monitored by UV absorption at λ =280 (A280). Immediately after collection, pH of each fraction was adjusted to 7 with ~2M Na_2PO_4. The antibody concentration was determined by measuring A280 (OD 280 of 1 corresponding to approximately 0.75 mg/ml of purified Ab). The Abs were stored at 4°C for short-term and at –20°C for long-term storage.

- labelling of antibodies with biotin

Purified antibodies were dialysed against 0.1 M $NaHCO_3$ (pH 8.2-8.6) in order to remove glycine. Glycine needs to be removed because it inhibits biotinylation reaction. To 1 mg/ml of antibody, 40µl of biotin succinimidyl ester stock solution (10 mg/ml in DMSO) was added for every ml of antibody. After incubation for 1h at RT unbound biotin was removed by gel filtration on PD-10 columns. The biotinylated antibodies were stored at 4°C for short-term use or aliquoted and stored at –70ºC.

- preparation of affinity columns

1g CNBr activated Sepharose was suspended and washed in low pH (1mM HCl in water), resuspended in PBS containing 3mg of a purified monoclonal antibody per 1 ml of Sepharose and incubated over night at 4°C under head-to-tail rotation. The ligand was removed and the remaining amine groups were blocked in 125mM Tris-HCl 1M glycine (pH 8) for several hours. The beads were then washed with PBS and column was set up. The column was primed with elution buffer, neutralized with PBS before use.

- enzyme linked immunosorbent assay (ELISA), sandwich ELISA

ELISA-microtiter plates Immulon2 (Dynex) were coated o/n at 4ºC with 1-10 µg/ml protein in PBS. The plates were thoroughly washed with PBS and blocked for 1h at 37ºC with 4%BSA in PBS. After washing with PBS, the desired antibody, protein or cell culture supernatant was applied for 1h at RT. The plates were then washed 3 times with PBS and secondary HRP linked antibody (anti-rat) or HRP linked straptavidin were added for 30 min. After washing (3 times with PBS) the ABST substrate solution was added (100µl/well) and incubated at RT for 20-30 min. ABTS produces a green coloured product upon reaction with HPR, which can be measured photospectrometrically at 405nm.
For sandwich ELISA experiments the plates were coated with purified monoclonal anti-LAG-3 Abs at 2µg/ml.

For isotyping of monoclonal antibodies, the plates were coated with soluble LAG-3D4t (2μg/ml in PBS), and anti-rat IgG2a or anti –rat IgG2b (Pharmingen) were used as secondary reagent, followed by Streptavidin –HRP.

-SDS-PAGE

Polyacrylamide separating gel was prepared according to the following protocol:

Separating gel percentage	12%	10%	7.5%	stacking gel (5.0%)
Deionized water	4.35ml	4.9ml	5.6ml	2.87
1.5M Tris-HCl,pH8.8 1 M Tris-HCl,pH6.8	2.5ml	2.5ml	2.5ml	 0.5ml
10%(w/v) SDS	100μl	100μl	100μl	40μl
40% Acryl./Bisacryl	3.0ml	2.5ml	1.75ml	0.5ml
10% APS	100μl	100μl	100μl	40μl
TEMED	4μl	4μl	6μl	4μl
Total	10ml	10ml	10ml	4ml

Separating gel was poured and left to polymerize for one approx. 1 hour at RT. Then the stacking gel was prepared. Samples were loaded on a gel in 1x loading buffer. For reducing conditions DTT at final concentration of 100 mM was added to the samples. The samples were subjected to electrophoresis in Tris-Glycine buffer at 200V for 45min-1 hr along with BENCHMARK™size marker in the Bio-rad minigel apparatus.

- analysis of polyacrylamide gels with GELCODE® Blue Stain Reagent

The polyacrylamide gels were washed 3 times for 5 minutes with deionized water, then immersed in GelCode® Blue stain reagent, and incubated at RT for 1 hr gently rocking. After staining the gel was washed in water at RT for 1 hr, the gels were scanned to save the image.

- preparation of the total protein lysates from cells

Cells were lysed in the lysis buffer at the concentration of 10^7-10^8cells per ml of lysis buffer by repeated pipetting and 30 min. incubation on ice. Then the insoluble fraction was spun down (20 000 xg, 10 min, at 4ºC) and the supernatant was collected.
Lysis buffer included: 1% NP-40, 50 mM Tris pH 7.8, 150mM NaCl, 1mM EDTA and Complete® protease inhibitors cocktail.

- Western blot analysis

After protein samples were separated on an SDS-polyacrylamide gel, the electrophoretic transfer to the membrane was done in the Tris-glycine transfer buffer containing 20% methanol for 1 hr at 350mA. After blocking for 1 hr with 4% non-fat milk in PBS at RT, the membrane was incubated with the first step antibody (polyclonal rabbit anti-LAG-3) for 1h at RT and washed in the same buffer for 15 min. The secondary antibody (goat anti-rabbit labelled with horse radish peroxidase (HRP)) was added for 45 min. at RT and after washing 3 times for 10 min. with PBS the blot was developed with the ECL Kit.
The chemiluminescence was recorded using LAS-1000 Imaging System (Fujifilm).

- saturated ammonium sulfate (SAS) precipitation

Splenocyte culture supernatants were centrifuged 30 min at 20 000xg at RT and transferred to a fresh tube. Saturated ammonium sulfate (SAS) was added slowly with stirring up to 45% (v/v). Precipitation was carried out at 4 C for 2 hrs. Samples were centrifuged again 30 min at 20 000 x g at RT. Precipitates were dissolved in PBS and used immediately or stored at –20°C.

- production and purification of soluble LAG-3

After a 3-week selection period, the expression of recombinant proteins was induced in stably transfected SL-3 cells (see below) by adding $CuSO_4$ to a final concentration of 1mM. Three days later (longer induction times are not recommended, since prolonged Cu

treatment leads to a decrease in cell number (Bernard et al., 1994)), cell lysates and supernatants were tested for the presence of soluble LAG-3 with SDS-PAGE and immunoblotting.

For large-scale cultures roller-bottles were used (450-500ml culture per roller bottle).

LAG-3D4t cultures reading the cell density of 5x 10^6 /ml were induced with $CuSO_4$ for 2-3 days and the supernatant was harvested by centrifugation (10 min at 2100g, 4°C) followed by 0.45 μm membrane filtration. At this point protease inhibitors (from 500x stock) were added to the supernatant to prevent degradation of soluble protein. Inhibitors stock solutions were: EDTA 500 mM, Leupeptin 0.250 mg/ml, Pepstatin 0.350 mg/ml, phenylmethylsulfonyl fluoride (PMSF) 50 mg/ml. The supernatant (now also containing sodium azide (0.05%) to prevent bacterial contamination) was then loaded on an anti-LAG-3 antibody column (monoclonal Ab G40) at 1-2 liters per day flow rate. The affinity column was extensively washed with PBS. Bound material was eluted with Glycine-HCL buffer of pH 3.2. Fractions were immediately neutralized with Na_2PO_4 (app. 2M) to bring pH up to 7.0, and stored at 4°C.

For production of de-glycosylated LAG-3D4t, tunicamycin (from Streptomyces sp. T7765, Sigma), at final concentration of 1μg/ml was added to the cultures at the point of induction. Tunicamycin was diluted from stock cultures prepared by dissolving 5 mg/ml tunicamycin in alkaline water (pH 11) and stored at -20 °C in 0.25 ml aliquots. Purification of the de-glycosylated protein was carried out as for glycosylated LAG-3D4t.

-immunocytochemistry

First, the poly-D-lysine glass slides were prepared in the following way: cover slips were washed with ethanol, dried and flooded with poly-D-lysine (0.1 mg/ml in PBS from 10mg/ml stocked prepared in water) for 30 min at RT. The excess of poly-D lysine was removed by aspiration, the slips were rinsed with cell culture grade H_20 and dried. Everything was done under sterile conditions.

A5 or A5LAG-3 cells were grown on poly-D lysine coated glass slides to 60-80% confluency. The cells were fixed in 1.8% paraformaldehyde 10 min at RT washed with PBS and blocked with 2% BSA 10% FCS PBS for 10-20min at RT. Incubation with the primary reagent; biotinylated Abs in blocking buffer was carried out for 1hr (RT). The

cells were washed with PBS and streptavidin Texas Red was used as secondary reagent (1hr at RT). After PBS wash, the slips were placed on microscope slides and viewed under fluorescent microscope (Nikon).

3.2.3. Cellular biology techniques

- cell culture

For cell culture work all the media and reagents were filtered through 0.2μm Millipore filters before use. Lymphocytes isolated from spleens and lymph nodes of mice and mouse cell lines were cultured in SF-IMDM supplemented with 2% FCS, in this work referred to as normal growth medium, in 95% humidity, 5% CO_2 and 37ºC.
For culturing of A5 cells hygromycin at 300 μg/ml was added to the growth medium.
For culturing of LAG-3 transfected cell lines puromycin at 2.5 μg/ml was added to the growth medium.
For culturing of sorted cells Ciproxin was added (at final concentration 10 μg/mL) to the normal growth medium to prevent contamination.
For freezing of mouse cells 40% FCS 10% DMSO normal growth medium was used.

- transfection of cell lines

A5 (HA specific CD4 T cell hybridoma) cell line was transfected using DMRIE-C Reagent ® according to manufacturer's instructions.

- transduction of A20 cells

GP-E 86 packaging cell line (Markowitz et al., 1988) (provided by prof. Antonius Rolink, University of Basel) was stably transfected with pLXS-puro vector (Backstrom et al., 1996) carrying murine Lag-3 in the following way: 1.6 x 10^6 cells were plated in 75 cm^2 flask a day be fore to reach 80% confluency on the day of transfection. 12 μg of plasmid DNA were diluted in 600 μl of serum free medium (DMEM) and separately 36 μl of Lipofectamine® Reagent were diluted in 600 μl of serum free DMEM, the two solutions were combined and incubated at RT for 30 min. Cells were washed with serum free

medium. 4.8 ml of serum free DMEM were added to the tube containing lipid-DNA complexes and the mixture was overlayed onto washed cells, followed by 5 hrs incubations at 37 °C. Then 6 ml of 20% FCS DMEM was added to the cells (without removing transfection mixture). Next day the medium was replaced by normal growth medium and the puromycin selection (2.5 μg/ml) was started day after.
Stably transfected GP-E86 cells were grown to 60-80% confluency (without puromycin) and irradiated (3000 rads), the growth medium was changed and A20 cells were co-culture with irradiated GP-E86 cells for 2-3 days, followed by transfer to a new flask and puromycin selection in the normal growth medium.

- cell culture and transfection of *Drosphila melanogaster* cells

Schneider cells (SL-3) were grown in the Sf- 900 II SFM supplemented with 1% FCS at densities between $5x10^5$ per mL and $2x10^6$ per mL at room temperature. SL-3 cells were transfected with 1.5 μg of pRmHa-3LAG-3D4t or pRmHa-3LAG-3D2t together with 0.1 μg of phshspuro-2 DNA plasmid (containing the puromycin resistance gene under the control of heat shock promoter (Steller and Pirrotta, 1986)) using Lipofectin® reagent according to the manufacturer's protocol. Transfections were performed in 24-well plate and puromycin selection (5μg/ml) was started two days later. Puromycin was chosen as a selection marker, rather than commonly used hygromycin B or geneticin, because it reduces the time required for selection to 2 weeks (compared to 3-4 weeks for hygromycin B and 5-6 weeks for geneticin (Towers and Sattelle, 2002)).
Colonies of drug-resistant cells could be seen about 1 week later. Cells were expanded and kept in culture or frozen in SF-900 II SFM containing 30% FCS and 10% DMSO.

- generation of B cell hybridomas by cell fusion

Lymph node single cell suspension taken from an immunized rat and the SP2/0 myeloma cell line were washed with pure IMDM and spun down together in one tube. 1-2 large flasks of SP2/0 cells in logarithmic growth phase per lymph node were used per fusion. 1.0 ml PEG1500 was added to cells within 1 min. while shaking in a 37ºC water bath. After another 90 seconds of shaking, the suspension was diluted with 5ml IMDM drop-

wise over the course of 90 seconds. All was done in 37ºC water-bath in 50ml Falcon tube. The tube was finally filled up with IMDM and after centrifugation, the cells were resuspended in 1 liter of HAT, IL-6, and 2%FCS SF-IMDM medium and plated out in 96 well plates (200 μl/well).

- preparation of cell suspension from mouse lymphoid organs

Freshly isolated spleen(s) and/or lymph nodes were transferred on a nylon net (Nitex) in petri dish with PBS 2% FCS. The cell suspension was made by gently pushing the organs though the net. The cell suspension was transferred into a 15mL Falcon tube and pelleted by centrifugation at 300g for 5-10 min. If the cells were used for biological assay, the procedure was done under sterile conditions.

- lysis of erythrocytes

Splenocytes were collected by centrifugation (10 min, 300g) and resuspended in normal growth medium (1 ml of per 1 mouse spleen). 4 ml of Gey's solution was added per 1ml of cell suspension, the mix was incubated at room temperature for 1 min., after which 5 ml (per 1 ml of original cell suspension) of normal growth medium was added to the tube. This treatment is based on hypotonic shock to which erythrocytes are very sensitive.

- preparation of dendritic cells from lymphoid organs

The total spleen or LN cell populations of wild-type mice (Balb/c strain) were enriched for dendritic cells by density centrifugation using Opti-prep® reagent.
Peripheral lymph nodes (inguinal, popliteal, axillar and auricular) and spleens of Balb/c mice were collected. Tissues were cut into small pieces and digested twice for 30 min. at 37°C in IMDM supplemented with 5% FCS and 100μg/ml collagenase D (37 °C room, gentle shaking). Cells were recovered, passed through a mesh (to get single cell suspension), centrifuged (5 min., 300g 4°C) and resuspended in HBSS (modified HBSS without Calcium and Magnesium) supplied with 25mM Hepes and 5mM EDTA (6 ml for 4-5 spleens). 3 ml of cell suspension was mixed well with 1 ml of OptiprepTM reagent

and placed in a 14 ml Falcon polycarbonate tube, 5 ml of 1 to 4.2 dilution of OptiprepTM (1 unit Optiprep to 4.2 units Optiprep dilution buffer) was then layered over cell suspension followed by 3 ml of HBSS. The gradient was centrifuged at 600g for 15 min. at RT without a brake. The layer between HBSS and OptiprepTM dilution (DC-enriched phase) was collected and washed with 5% FCS IMDM. The enrichment varied between 30 to 60 fold. The obtained cells were cultured or stained with antibodies for flow cytometric analysis and/or cell sorting.

- surface staining of cells for FACS analysis and purification

Staining of cells was performed in 96-well V-bottom plates. For all stainings involving B cells, the cells were incubated with anti-mouse CD16/CD32 (2.4G2) antibody at 10μg/ml for 5-10 min on ice to block Fc receptors. 10^5-10^6 cells per well were stained with the given antibody at the pre-determined dilution (usually 10-20μg/ml was optimal) in 2%FCS PBS on ice for 5-15 min. MAbs were used alone or in various combinations. They were either directly labelled with Fluorescein Isothiocyanate (FITC), R-Phycoerythrin (PE), Peridinin Chlorophyll (PerCP) or Allophycocyanin (APC)- or were biotinylated. The latter ones had to be revealed by secondary staining with Streptavidin –PE or FITC. After each incubation cells were washed once with 2%FCS PBS. Until analysis was undertaken, samples were kept on ice. Analysis was done on a FACScalibur flow cytometer (Becton Dickinson).
If cells were to be sorted and cultured, all procedures were done under sterile conditions. For larger cell numbers (10^6-10^8) 14 ml Falcon tubes were used. Cells were suspended at a concentration of $5x10^6$ per ml for FACS and $30x10^6$ per ml for cell sorting, and filtered through a nylon mesh. Cell sorting was performed on FACSAria (Becton Dickinson).

- MACS purification/enrichment of cells

MACS (Magnetic Cell Sorting) LS Separation Columns were used for enrichment and/or purification of T cells or B cells according to the manufacturer's instructions (Miltenyi Biotec). Biotinylated primary antibodies (CD4 or CD19, respectively) were used for labelling, followed by Streptavidin MicroBeads.

- CFSE labelling

CFSE was dissolved in DMSO to 5mM (stock solution), aliquoted (10 µl per tube) to avoid repeated freezing and thawing which may degrade the reagent and stored at- 20°C. For labelling, cells were resuspended in 0.1 % BSA/PBS at 10^7 cells/mL and CFSE at a final concentration of 5µM was added to them. The cells were then incubated at 37°C (water bath) for 10 min. The reaction was stopped by adding 10-20 x volume of 0.1 % BSA/PBS. The cells were centrifuged for 10 min at 300g, washed once with the growth medium (room temperature) and used right away for culture.

- stimulation of T cells

Total splenocytes, sorted T cells or A5 cells were stimulated with anti-CD3 Ab (145.2C11) alone or in combination with anti-CD28 Ab (37.51). The 24 or 96 flat bottom plates were coated with the antibodies at 2µg/ml in PBS for 2-3 hours at room temperature or overnight at 4°C, and washed once with PBS before plating cells. Cells were plated at $2x10^6$ per well (24 well plates) or 0.2 - 0.3 $x10^6$ per well (96 well plates). For antigen specific stimulations A20 B cell lymphoma was used as antigen presenting cells. After γ-irradiation (30Gy) A20 cells were washed once with the growth medium and loaded with various concentrations of HA peptide (SSFERFEIFPK) for 2hrs at 37°C.

- stimulation of B cells

Purified B cells were plated 0.1 mln cells/well (96 well flat bottom plates) in normal growth medium supplied with 2%FCS. For stimulations CpG 1826 (Ballas et al., 2001) at final conc. 0.3 µg/ml , LPS at final conc. 25 µg/ml , purified anti-mouse CD40 mAb (FGK-45) and anti-mouse Ig κ L chain mAb (187.1) (10 µg/ ml each Ab) supplemented with IL-4, were used.

- stimulation of dendritic cells

The cell population enriched for dendritic cells or sorted dendritic cells were plated in round bottom 96 well plates, 1 $x10^5$ cells/well (spleen DCs) or 1-2 $x10^4$ cells/well (LN DCs). Final concentrations of stimuli were: LPS -25 μg/ml, anti-CD40 antibody (FGK-45) -10μg/ml, TNF- 50 ng/ml, CpG 1826 - 0.3 μg/ml.

- regulatory cell assay

Sorted CD4+ CD25- CFSE labelled effector T cells (2.5 $x10^4$), 1 x 10^5 irradiated (20 Gy) WT splenocytes, and sorted CD4+CD25+ regulatory T cells (2.5 $x10^4$) or non-labelled effector cells (2.5 $x10^4$) were plated in round bottom 96 well plates in the normal growth medium. Cultures were stimulated with various doses of soluble anti-CD3 Ab (145.2C11) for 4 days. Effector T cells activation was determined by CFSE dilution analysis.

- removal of dead cells with Ficoll-Paque

Cells were resuspended in normal growth medium at 1-5 $x10^8$ cells/ml and 5ml of Ficoll-Paque solution was layered under the cell suspension. The samples were then centrifuged for 15 min at 800xg at RT without brake. The live cells floating on the top of the high-density solution were transferred to another tube, washed twice with the growth medium and used for subsequent procedures.

- OT-II transgenic T cell activation assay

RBC depleted splenocytes and LN cells from OT-II mice were pooled and stained with anti-CD4 FITC Ab (for positive selection of transgenic CD4 T cells), anti-CD8 PercP Ab (to make sure that all CD8 cells, that also could respond to OVA peptide are excluded) and anti-CD44 PE (to exclude activated cells). The CD4+ cells were sorted and labelled with CFSE (as described above). For activation, LAG-3 positive or LAG-3 negative APCs were loaded with various concentrations of 323-339 OVA peptide for 2 hours (50 000 cells per well, in 96-well round bottom plates), and 25 000 of CFSE labelled reporter

CD4 T cells was added per well. T cell proliferation was monitored by CFSE dilution measurement for 6 days.

3.3. Mice

BalbC	HARLAN Italy, San Pietro al Natisone, Italy
C57BL6	HARLAN Italy, San Pietro al Natisone, Italy
LAG3 knock-out	Dr. D. A. Vignali, Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN
OT-II	Charles River Laboratories, France
RAG/CγC dko	Institute for Research in Biomedicine mouse facility, Bellinzona

In all the experiment the mice we age and sex matched.

3.3.1 Animal techniques

- collection of organs

6-12 weeks old mice were sacrificed by CO2 inhalation and spleen and LN (mesenteric, inguinal, axillar and popliteal) were removed.

-reconstitution of lymphopenic mice

RAG/CγC double ko mice were used as recipients. These mice have no B, T nor NK cells. 5 x 10^6 of total C57BL6 LN cells were injected in tail vain of sublethally (400 rads) irradiated recipient mice.

- LPS injections

5ug of LPS (in 200ul of PBS) per mouse was injected intraperitonally (i.p.).

4. RESULTS AND DISCUSSION

4.1. Generation and characterization of monoclonal antibodies against mouse LAG-3

In order to study the expression pattern and function of LAG-3 we produced a number of monoclonal antibodies (mAbs) against mouse LAG-3. Lewis rats were immunized by injection into foot pads with LAG-3 transduced rat myeloma Y3 cells. Specific antibody-producing hybridomas were selected by flow cytometry (surface staining of LAG-3 expressing cell line) and by ELISA with LAG-3hIgG1 fusion protein as antigen. MAbs produced by selected clones were then epitope mapped using recombinant LAG-3 molecules consisting of either D1 to D2 (D1-D2) or D1 to D4 (D1-D4) domains, as shown in Figure 4.1.1, and grouped into 2 specificity categories: D1-D2 and D3-D4.

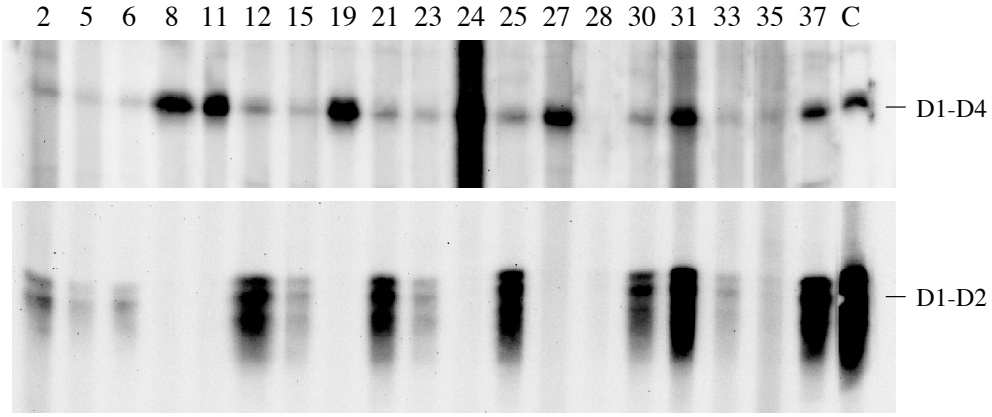
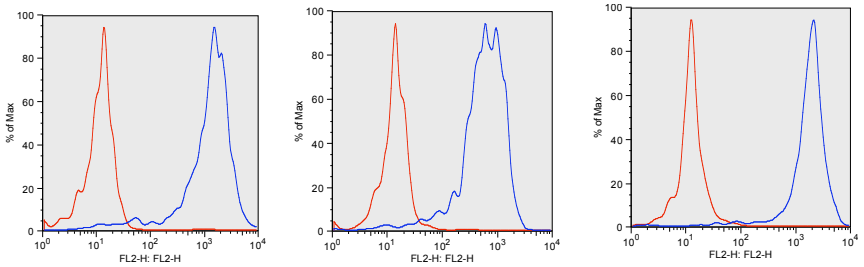


Figure 4.1.1. Screening of anti-LAG3 hybridomas. Recombinant LAG-3 molecules composed of D1 to D4 (D1-D4) or D1 to D2 (D1-D2) domains (for more information see section 4.2) in the form of *Drosophila* culture supernatant (D1-D4) and *Drosophila* cells lysate (D1-D2; D2l truncation), respectively, were separated on non-reducing 10% PAGE. The blots were probed with hybridoma supernatants (various hybridomas are represented by numbers indicated at the top), followed by goat anti-rat Ig HRP linked conjugate. Rabbit polyclonal anti-mouse LAG-3 Ab, followed by goat anti-rabbit HRP conjugate were used as a positive control (C). Sample blots are shown.

Twelve hybridomas were chosen for further analysis; G15, G21, G23, G30, G31, G35, G37, G38 which are specific for D1-D2 region and G19, G24, G27 and G40 specific for D3-D4 region. Some of them (for example G15 and G35) did not perform well in western blot, likely because they recognize conformational epitope that is lost upon denaturation of the



G 15 G 19 G 35

Figure 4.1.2. Staining of activated lymph node cells with purified anti-LAG-3 mAbs G15, G19 and G35. The cells were activated on immobilized anti-CD3 for four days. For analysis the samples were gated on CD8 T cells from wild type (in blue) or LAG-3 deficient (in red) mice.

a)

Biotinylated Abs (detection)

Purified Abs (presenting)

	15	21	23	24	30	31	35	37	38	40
15	–	+	–	+	w	–	+	vw	vw	+
19	+	w	+	+	+	+	+	+	+	+
23	–	+	–	+	w	–	+	vw	vw	+
24	+	–	+	–	+	+	w	+	+	–
30	–	w	–	+	–	–	+	vw	vw	+
31	–	vw	–	+	–	–	+	vw	vw	+
35	+	–	+	+	+	+	–	+	+	+
37	–	w	–	+	w	–	+	–	–	+
38	–	w	–	+	w	–	+	–	–	+
40	+	–	+	–	+	+	w	+	+	–
27	+	vw	w	+	+	+	vw	+	+	+

b)

EP 1 -G24/G40
EP 2 - G15/23
EP 3 - G 21
EP 4 - G19
EP 5 - G27
EP 6 - G37/G38
EP 7 – 35
EP 8 - 30/31

Figure 4.1.3. Sandwich ELISA was performed with purified monoclonal antibodies (indicated on the left) presenting the soluble LAG-3 (D1 to D4). Biotinylated antibodies (top) were used for detection. w- weak signal, vw- very weak signal.

samples. The isotypes of mAbs were determined by ELISA (not shown). Two of them: G31 and G37 Abs are of IgG2b isotype, the other 10 Abs are IgG2a.

The monoclonal antibodies were purified, biotinylated and their specificity was ascertained by the ability to stain αCD3 and αCD28 Abs- activated splenocytes from wild-type, but not from LAG-3 deficient mice, as exemplified in Figure 4.1.2. Furthermore, sandwich ELISA results showed that at least 8 different epitopes were recognized by various monoclonal Abs (Figure 4.1.3).

The obtained antibodies were also tested in immunocytochemistry. T cell line stably transfected with LAG-3 (A5LAG-3 cells, see below) was plated on cover slips, paraformaldehyde (PFA) fixed and stained with various antibodies. In this type of application G24 and G37 Abs were working well (Figure 4.1.4)

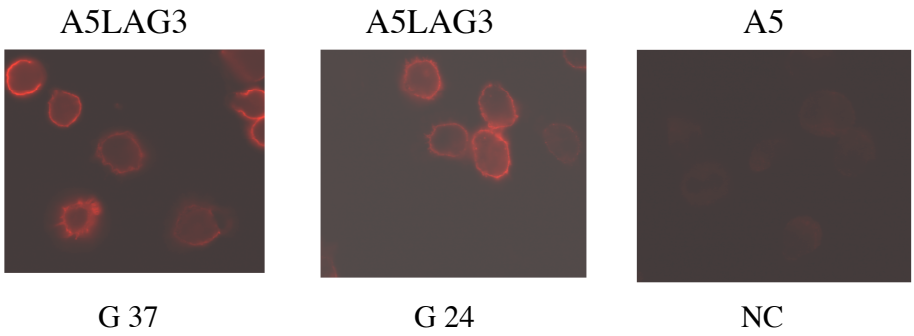


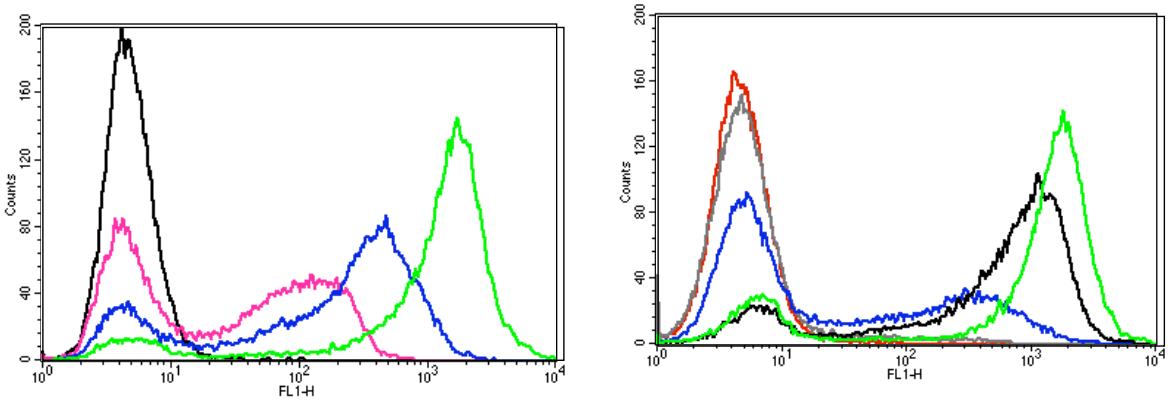
Figure 4.1.4. Immunocytochemical analysis of LAG-3 expressing T cell line, A5LAG-3 (left and middle panel). Biotinylated G37 and G24 Abs were used for detection, followed by streptavidin Texas Red. Non -transfected A5 cells served as negative control (NC), right panel.

It was also analyzed if the generated monoclonal antibodies were functionally active. For this purpose A5 cells, an HA-peptide specific T cell hybridoma carrying GFP gene under the control of minimal IL-2 promoter (Andersen et al., 2001) was used. These cells turn green upon activation, which can be easily detected by FACS. Figure 4.1.5 shows both time course and concentration curve of A5 cells' response to proliferation stimuli.

LAG-3 positive variant of T cell hybridoma (A5) was made by stably transfecting the cells with a plasmid harbouring LAG-3 cDNA sequence under the control of EF-1a promoter. A5 LAG-3 cells express high levels of LAG-3 on their surface (Figure 4.1.6).

a) time course

b) dose response



FL-1

FL-1

Figure 4.1.5. Kinetics and dose response of A5 cells stimulated with αCD3 Ab or by specific antigen. A5 cell activation can be monitored by the increase in GFP fluorescence. (a) A5 cells were stimulated with immobilized αCD3 Ab and analyzed at various time points: 0 (black), 4 (pink), 8 (blue) and 20 (green) hours. (b) A5 cells were stimulated for 16 hrs with HA peptide pulsed A20 cells (B cell lymphoma). Various concentrations of peptide were used: 0 (red), 0.1 (grey), 1 (blue), 10 (black) and 100 (green) μg/ml.

A5

A5LAG-3

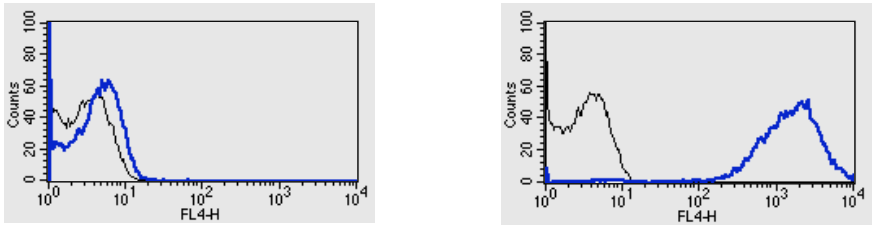


Figure 4.1.6. The expression level of LAG-3 on the surface of A5LAG-3 cells. A5 cell were stably transfected with Lag-3, LAG-3 surface levels were analyzed by staining with biotinylated mAb G15, followed by Streptavidin-APC. LAG-3 - blue line, negative control (secondary reagent only) - black line.

When A5 and A5LAG-3 cells were stimulated with APCs pulsed with the relevant antigen, we observed that the T cells with LAG-3 on their surface showed poor activation as compared to WT A5 cells (Figure 4.1.7, pink line versus dark blue line). This result is consistent with the results of similar experiments done by others (Workman et al., 2002a). The inhibitory effect was very strong (more than 95%), and decreased slightly (to about 60 %) with increased HA peptide concentrations used for loading of APCs.

Subsequently, it was tested if mAbs against LAG-3 can counteract the activity of ectopically expressed LAG-3. Indeed, the majority of monoclonal antibodies were able to efficiently neutralize the inhibitory effect of surface LAG-3. Upon addition of monoclonal antibodies, the activation levels of A5LAG-3 cells came close to that A5 cells, as shown in Figure 4.1.7. The strength of 'neutralizing effect' did not strictly correlate with the epitopes recognized by mAbs. The D3-D4 mAbs G24 and G40 were not very efficient in neutralizing LAG-3 function, but the D3-D4 mAbs G19 and G27 were as efficient as all D1-D2 Abs tested.

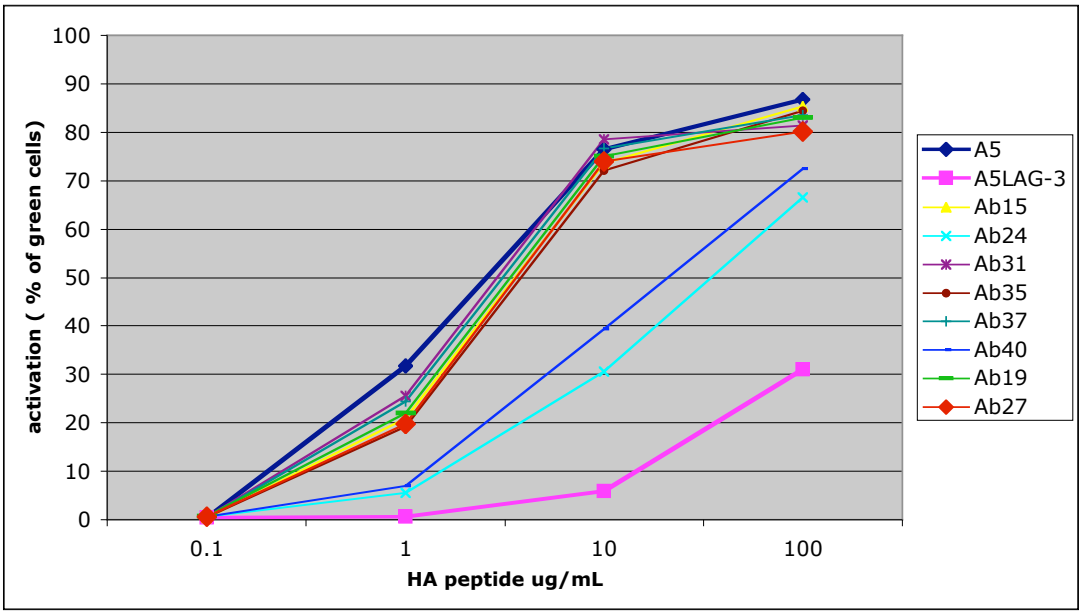


Figure 4.1.7. Monoclonal antibodies are able to neutralize inhibitory effect of surface LAG-3 on T cell activation. A20 cells were pulsed with HA peptide at indicated concentrations and used to activate A5 or A5LAG-3 cells in the absence or presence of various mAbs. The activation of A5 cells was not affected by mAbs. The effect of mAbs on the activation A5LAG-3 cells is shown.

Altogether, the above results indicate that the new mAbs against LAG-3 we generated are highly specific and are functionally active *in vitro*. They work in various types of assays and hence are ideal for investigating LAG-3 expression and function in the immune system.

4.2. Production of soluble LAG-3 for structural studies

As insect cells are known to be suitable hosts for heterologous protein expression and production, we chose Schneider cell line (Schneider, 1972), a cell line derived from primary cultures of late-stage of *Drosophila melanogaster* embryos, for the production of soluble LAG-3.

It was first tested if the LAG-3 consisting of all four extracellular domains (D4t) and its shorter truncation (D2t) consisting of the two outmost domains can be expressed in *Drosophila* SL-3 cells. The coding sequences for D4t (nucleotides 357-1769) and D2t (nucleotides 357-1122) were amplified from thymus cDNA by PCR and cloned into pRmHa-3 vector. The important feature of pRmHa-3 vector is the metallothionein (MT) promoter, which contains heavy metal response elements. This allows for inducible expression of protein under investigation, upon addition of cadmium or copper, the latter being less toxic to the *Drosophila* cells (Bunch et al., 1988).

SL-3 cells stably transfected with pRmHaLAG-3D4t or pRmHaLAG-3D2t plasmid were treated with CuSO4 for 3 days to induce the production of recombinant proteins. Culture supernatants as well as cell pellets were collected. The supernatants and cell lysates were treated with protease inhibitors to prevent protein degradation. Western Blot analysis was performed to check the levels of the recombinant LAG-3 production. As shown in Figure 4.2.1, D4t truncation was present in both in cell lysate and in culture supernatant, while D2t was produced in the cells but could not be secreted. D4t protein migrated with apparent molecular weight of 53 kDa and D2t product of 25 kDa.

Because LAG-3 D2t was retained inside the cells, two additional short truncations (D2s encoded by nucleotides 357-1092 and D2l encoded by nucleotides 357-1157) were constructed. As for D2t, although both of them were produced in SL-3 cells, they were not secreted into the media (Figure 4.2.2). This indicated that the shorter truncated forms of LAG-3 do not fold correctly and would not be suitable for further analysis.

At this point we focused on the longer version - D4t of recombinant LAG-3. Its presence in the SL-3 culture supernatant facilitated subsequent purification.

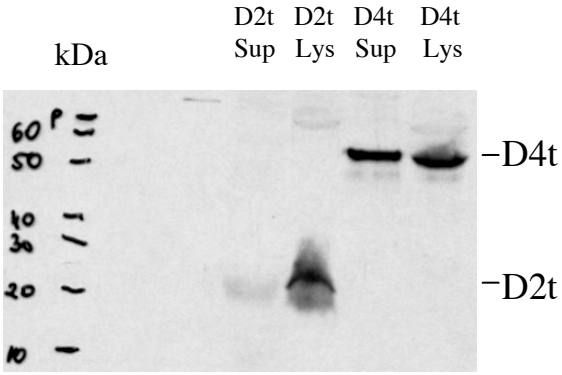


Figure 4.2.1. Western blot analysis of the supernatants (Sup) and cell lysates (Lys) of pRmHa LAG-3D4t or pRmHaLAG-3D2t stably transfected SL-3 cells after $CuSO_4$ induction. Samples were separated on 12 % PAGE under reducing conditions. The blot was probed with rabbit polyclonal anti-mouse LAG-3 Ab, followed by goat anti-rabbit HRP liked Ab.

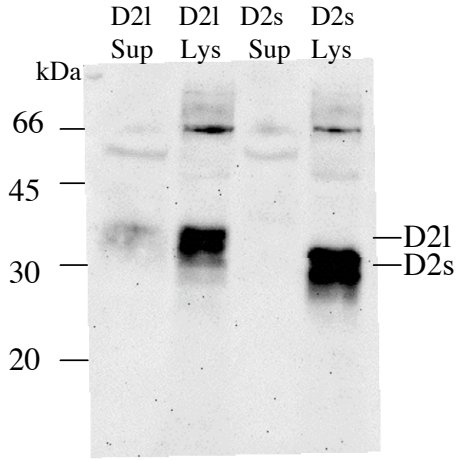


Figure 4.2.2. Western blot analysis of the supernatants (Sup) and cell lysates (Lys) of pRmHa LAG-3D2s or pRmHaLAG-3D2l transfected SL-3 cells after $CuSO_4$ induction. Samples were separated on 12 % PAGE under reducing conditions. The blot was probed with rabbit polyclonal anti-mouse LAG-3 antibody, followed by goat anti-rabbit HRP linked Ab.

We chose to affinity purify soluble LAG-3 D4t using one of our monoclonal antibodies. Ab G40 (IgG 2a) was coupled to CNBr-activated Sepharose 4B to prepare the affinity chromatography column. The soluble LAG-3 D4t was produced in a batch-wise manner (1.5 L at a time) and loaded on the G40 affinity column. After each load the absorption to the column was ensured by Western blot as shown in Figure 4.2.3a. The level of absorption to the column varied slightly from batch to batch, possibly due to small differences in speed of loading of the supernatant and/or the amount of protein present in

the supernatant. After reaching saturation (equivalent to 11 Liters of SL-3 culture), affinity column was eluted with pH 3.2 Glycine-HCL buffer. The protein content of the eluted fractions was determined by measurement of Absorbance at 280 nm (A280). LAG-3 D4t elution profile is presented in Figure 4.2.3b. Approximately 50 mg of recombinant protein was obtained.

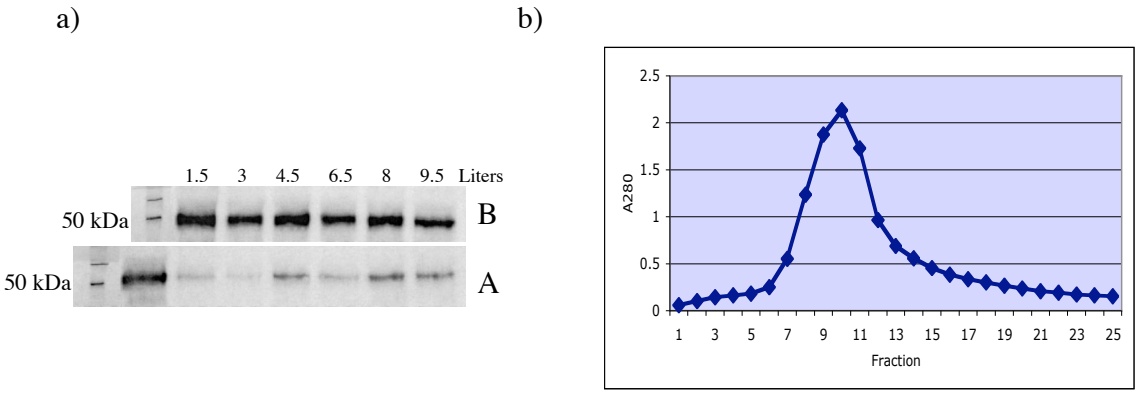


Figure 4.2.3. The production of soluble LAG-3. (a) The subsequent batches of LAG-3D4t expressing SL-3 cells culture supernatants (40 µl/lane) were analysed for presence of soluble LAG-3 by Western blot before (B) and after (A) affinity column. The blot was probed with rabbit polyclonal anti-mouse LAG-3 Ab, followed by goat anti-rabbit HRP linked Ab. (b) The elution profile of the column saturated with soluble LAG-3 is shown. A280 –Absorbance at 280 nm.

To determine the purity of affinity purified soluble LAG-3, samples of different fractions were subjected to 10% PAGE under reducing conditions followed by staining with GELCODE® Blue protein stain reagent. Figure 4.2.4 shows that this one step purification was very efficient; there is hardly any contamination detectable.
The whole procedure was repeated and additional 70 mg of the soluble LAG-3 D4t were obtained.

There are 5 potential N-linked glycosylation sites in murine LAG-3. And it is known that heterogenous glycosylation can prevent protein crystallization. To optimize the conditions for LAG-3 crystallization, we produced a non-glycosylated version of sLAG-3 in addition to wild-type glycosylated form of soluble LAG-3.
First, we tested if tunicamycin - a specific inhibitor of N-linked glycosylation would work in our culture conditions. As shown in Figure 4.2.5a, the pre-treatment of SL-3 culture before $CuSO_4$ induction is not required, but tunicamycin must be added at the

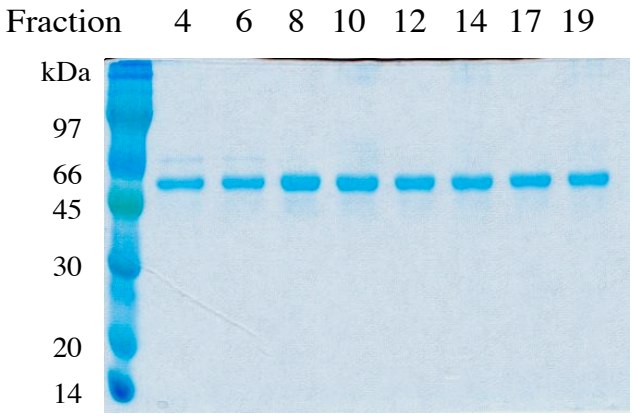


Figure 4.2.4. Affinity-purified soluble LAG-3 shows high degree of purity. 2 ug of total protein per fraction from affinity column-eluted fractions were subjected to 10% PAGE under reducing conditions. After separation gel was stained with GELCODE Blue Stain Reagent.

time of induction. When inhibitor was added 3 hrs after induction the glycosylated 'band' (the upper band, Figure 4.2.5a) was already visible. There is a clear shift in mobility of recombinant LAG-3 D4t after tunicamycin treatment, the non-glycosylated LAG-3D4t migrates at approximately 44 kDa (compared to 53 kDa of glycosylated version).

The above assessment of tunicamycin activity was performed with a quite high concentration of tunicamycin (5 µg/mL). It was then tested if, for a larger scale production, lower concentrations of tunicamycin could be used. Indeed, the concentration as low as 0.8 µg/ml worked well (Figure 4.2.5b). However, when 0.5 µg/ml of tunicamycin was used the level of inhibition showed variation between experiments (not shown). Finally, we decided on the1 µg/ml concentration; at this concentration no toxic effect was observed on sLAG-3 producing SL-3 cells, the problem often encountered using Baculovirus expression system involving Sf9 insect cells.

Next, the production of LAG-3D4t was repeated, this time in the presence of tunicamycin. Before the chromatography purification each batch was checked for efficient de-glycosylation (Figure 4.2.6, e.g. lane 7 to 9). As its glycosylated counterpart, non-glycosylated protein efficiently absorbed to the affinity column (Figure 4.2.6 lanes 1 to 6). After column saturation and elution in pH 3.2 Glycine-HCL buffer, the protein content of the eluted fractions was analysed on 10 % reducing PAGE, followed by GELCODE staining.

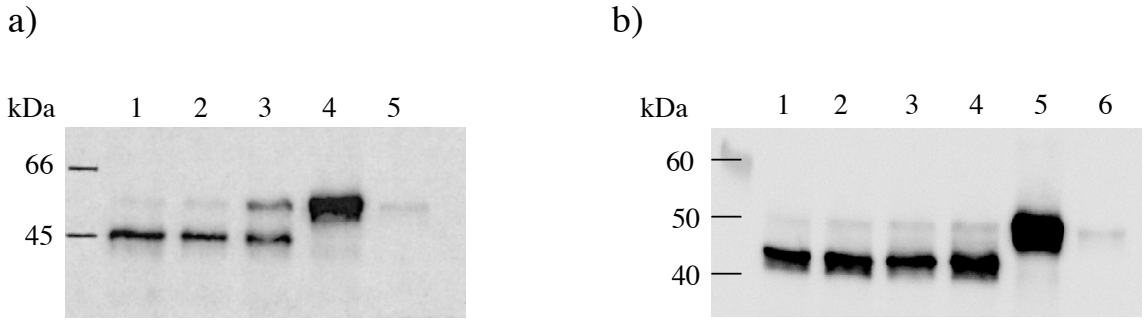


Figure 4.2.5. Production of non-glycosylated LAG-3D4t. (a) time course; lane 1- 5ug/mL tunicamycin 5 hrs before induction, lane 2- 5ug/mL tunicamycin at the time of induction, lane 3- 5ug/mL tunicamycin 3 hrs after induction, lane 4 - no tunicamycin, lane 5- no induction (b) concentration curve; lane 1- 5ug/mL, lane 2 - 3.3ug/mL, lane 3 - 1.7 ug/mL, lane 4 - 0.8 ug/mL, lane 5 - no tunicamycin, lane 6 - no induction. The supernatants were separated on 10% PAGE under reducing conditions. The blot was probed with rabbit polyclonal anti-mouse LAG-3 antibody.

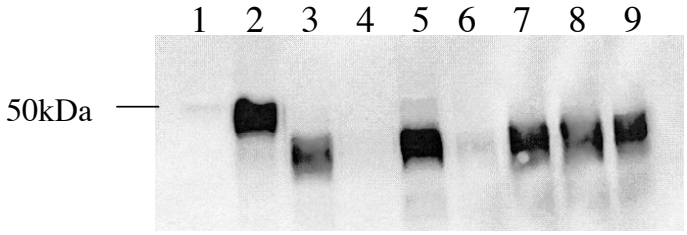


Figure 4.2.6. Western blot analysis of LAG-3D4t expressing tunicamycin treated *Drosophila* cultures. Lane 1 – no induction, Lane 2- with induction, Lane 3 – batch 1 before column, Lane 4 – batch 1 after column, Lane 5 – batch 2 before column, Lane 6 – batch 2 after column, Lane 7 – batch 3, Lane 8 – batch 4, Lane 9 – batch 5. All batches were tunicamycin treated. The supernatants were separated on 10% PAGE under reducing conditions. The blot was probed with rabbit polyclonal anti-mouse LAG-3 antibody.

Figure 4.2.7 includes the elution profile (a) and PAGE analysis (b) of the non-glycosylated soluble LAG-3 D4t. About 55 mg of pure protein were obtained from 12 liters of SL-3 culture. The 'large-scale' production procedure of non-glycosylated LAG-3D4t was repeated and additional 80 mg of purified protein were obtained (not shown).

a) b)

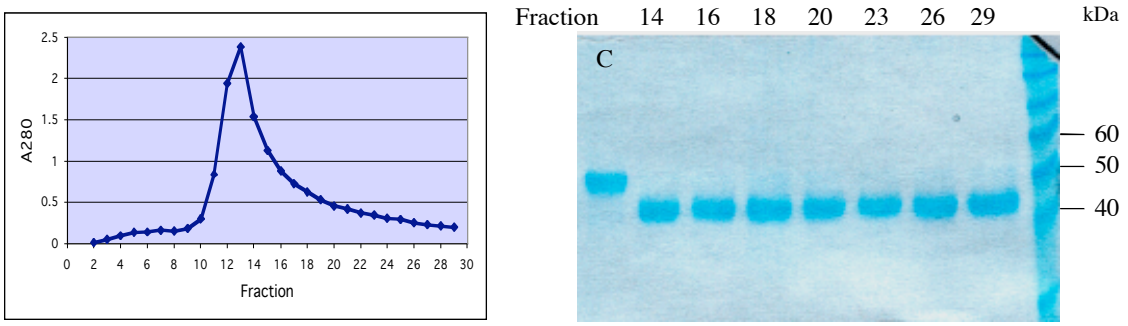


Figure 4.2.7. The production of non-glycosylated soluble LAG-3. (a) chromatography column protein elution profile (elution was carried out at pH 3.2) and (b) GELCODE Blue Stain Reagent staining. 2 ug of total protein per fraction from eluted fractions were subjected to 10% PAGE under reducing conditions. C- glycosylated soluble LAG-3 was used as control.

Overall, *Drosophila* SL-3 expression system established in the course of this work proved to be an efficient way to produce soluble LAG-3 (composed of four extracellular domains) in two flavors: glycosylated and non-glycosylated.

In general *Drosophila* cells are easy to maintain in culture and have the advantage over the mammalian cell expression system in that they grow optimally in room temperature and do not require CO_2 incubator.

The purified soluble LAG-3 proteins are now used in structural studies, which are done in collaboration with Dr. Roy Mariuzza (Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute, Rockville, USA).

Interestingly, soluble LAG-3D4t behaved as a dimer in size exclusion chromatography. The molar mass of LAG-3D4t calculated by amino acid composition is 43.7 kDa; however, the molar mass from MALDI (matrix assisted laser desorption/ionization) mass spectrometry was ~ 50.1 kDa, exhibiting several prominent peaks ranging from 49.4 to 51.6 kDa as a result of differences in the extent of glycosylation.

Unfortunately, all the efforts to crystallize this form of LAG-3 have failed, may be due to molecular heterogeneity caused by uneven glycosylation.

Pleasingly, however, the non-glycosylated soluble LAG-3 also behaved as dimer and respective MALDI analysis revealed, as expected, more homogenous molecular species (~ 44.9 kDa).

Attempts to crystallize non-glycosylated LAG-3D4t in its free and MHC class II bound forms are underway. If successful the detailed structural information can tell us more about LAG-3 itself as well as LAG-3–MHC class II interaction(s). In addition this structure may help us to understand how CD4, a well-known TCR co-receptor and a relative of LAG-3, binds to MHC class II – one of the key interactions in the immune system but of so low affinity that good quality complexes are extremely difficult to obtain.

Biochemical analysis of soluble protein showed that in solution LAG-3 forms stable dimers. This observation strongly suggests the presence of such dimers on cell surface. LAG-3 dimers on T cells could augment its avidity for MHC class II, or other ligand, and thereby enhance LAG-3 mediated signaling. This notion is supported by recent data by Li and colleagues. Their cross-linking studies of LAG-3-transduced T cell hybridoma showed that LAG-3 can form dimers and even oligomers at the cell membrane (Li et al., 2004). Human LAG-3 studies have previously also suggested that LAG-3 might be expressed as a dimer, but no biochemical support for this hypothesis has been presented (Huard et al., 1997).

Furthermore, as analysed by Western blot, soluble LAG-3 isolated from splenic culture supernatants (described in section 4.4 of this report) closely approached, in terms of molecular weight, the recombinant LAG-3 composed of 4 extracellular domains (glycosylated form). This observation supports the ‘proteolytic processing’ rather than ‘alternative splicing’ mechanism of soluble LAG-3 generation.

4.3. LAG-3 on naïve, effector and regulatory T cells.

Although there have been a number of reports about LAG-3, its function in the immune responses has remained enigmatic. The discrepancies between a human and a mouse system led to confusion. LAG-3 function as negative regulator of T cell activation concluded from studies with human cells was not confirmed in LAG-3 knock-out mice (Hannier et al., 1998; Huard et al., 1994b; Miyazaki et al., 1996). While the function for LAG-3 in NK cell killing, suggested from LAG-3 knock-out studies was not supported by evidence from human cells (Huard et al., 1998; Miyazaki et al., 1996). Also the later more detailed analysis of LAG-3 deficient mice was not consistent (Workman et al., 2004; Workman and Vignali, 2003).

Having new tools for LAG-3 analysis at hand, we decided to first re-examine the expression pattern and function of LAG-3 on mouse T cells.

4.3.1. Expression of LAG-3 on CD4 and CD8 T cells and its role in homeostasis

First, we looked at the LAG-3 expression on the surface of CD4 or CD8 T cells over time after CD3 triggering. Figure 4.3.1 shows the time course of LAG-3 expression on activated T cells. Freshly isolated mouse splenic and lymph node T cells do not express LAG-3 (not even approximately 2%, as reported by Workman and colleagues (Workman et al., 2002a)). It appears on the surface of T cells within 24 hours of CD3 engagement and reaches plateau on day 3 of activation (Figure 4.3.1 a and b).

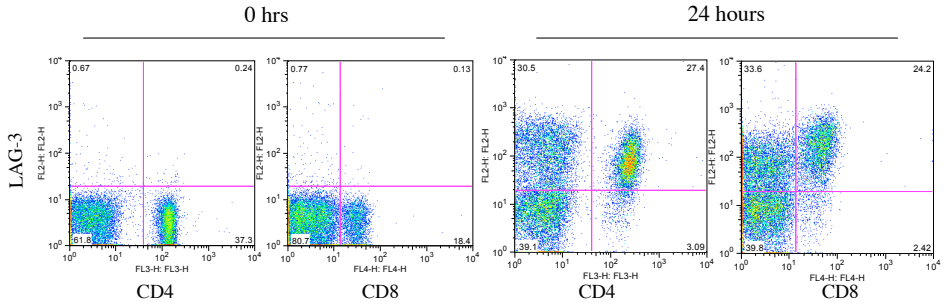
Furthermore, the levels of LAG-3 were slightly (approximately 2 fold) but consistently higher on CD8 T cells than on CD4 T cells. Similar observation has been reported by Huard and colleagues working with human PHA blasts (Huard et al., 1994a). This difference in LAG-3 expression pattern may be due to the fact that Lag-3 gene lies within CD4 locus (Bruniquel et al., 1997) and its promoter may be partially silenced when CD4 gene is activated. In addition, upon CD8 T cell triggering with the relevant peptide, LAG-3 co-capped with CD3 much more efficiently then in case of activated CD4 T cells (Hannier and Triebel, 1999). The observation that CD8 cells express more LAG-3 on their surface is difficult to reconcile with LAG-3 being the only MHC class II ligand.

But, LAG-3 function on CD8 T cells has not been extensively analyzed as the LAG-3 studies concentrated more on MHC class II restricted CD4 cells.

How could LAG-3 play a role in class I restricted responses?

Frederic Triebel, in his recent review, suggested the model of molecular interactions between a CD8 T cell and APC, in which he considers LAG-3 as independent co-receptor (Figure 4.3.2), supporting the idea that LAG-3 does not interfere with CD4-MHC class II interactions but rather directly inhibits T cell activation through intracellular signaling (Triebel, 2003). However, it is possible that a ligand other than MHC class II could be involved in LAG-3 function on CD8 cells.

a)



b)

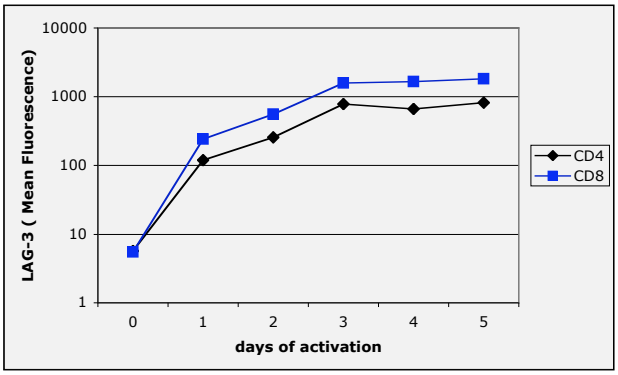


Figure 4.3.1. Activated T cells express LAG-3 on their surface. (a) Lymph node cells were activated on immobilized αCD3 Ab for 24 hours. Triple staining (CD4/CD8/LAG-3) before and after activation is shown. (b) Splenocytes were cultured on αCD3 coated plates for indicated times, staining was done as in (a) and the samples were gated on CD4 or CD8 T cells.

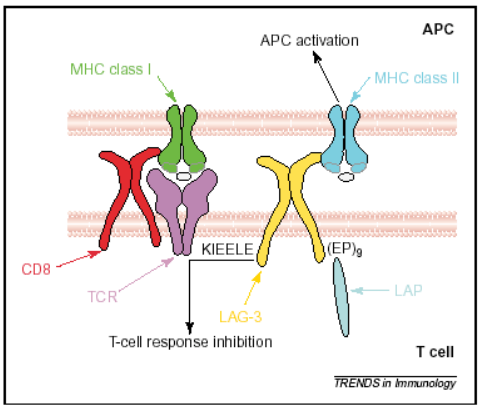


Figure 4.3.2. LAG-3 -MHC class II interactions. Shown are the consequences of LAG-3 interactions when a CD8 T cells meets an APC. Two motifs of the intraplasmic region potentially involved in signaling are shown (from (Triebel, 2003). In such scenario signaling downstream from LAG-3 would interfere with signaling pathways activated by TCR tiggering.

Then, we looked at the kinetics of LAG-3 down-regulation and asked if continuous stimulation is necessary for surface LAG-3 expression. In order to see how long LAG-3 persists on the surface of T cells after removal of proliferation stimulus, purified T cells were activated on immobilized anti-CD3 and anti-CD28 Abs for 3 days, then collected, washed and cultured in normal growth medium supplied with IL-2. As shown in Figure 4.3.3, after removal of the stimulus, T cells kept high levels LAG-3 on their surface for at least 24 hours; followed by about 50% loss after 4 days, and after 7 days going down to

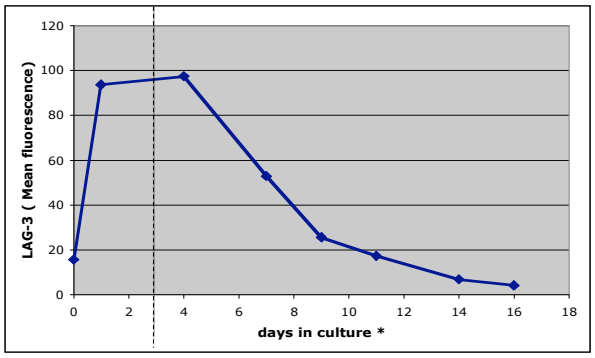


Figure 4.3.3. Surface expression of LAG-3 on activated T cells after removal of proliferation stimulus. Purified T cells were activated on immobilized anti-CD3 and anti-CD28 for 3 days and transferred to IL-2 supplied growth medium. The time of transfer is indicated by the dotted line.

the level before activation. Therefore, it can be concluded that continuous stimulation is required to maintain high levels of surface LAG-3.

Until now, all of the studies on murine LAG-3 demonstrating its presence on T cells were carried out *in vitro*. In an attempt to find LAG-3 *in vivo*, we looked at homeostatically expanding T cells. Using our new monoclonal antibodies we show that LAG-3 appears on the surface of T cells undergoing homeostatic proliferation in lymphopenic hosts. LN cells from normal mice were transferred into sublethally irradiated recombinase activating gene-2 and common cytokine receptor γ chain (RAG 2/CγC) double ko mice (Mazurier et al., 1999). RAG/CγC are suitable hosts for the lymphocyte reconstitution experiments, because they are completely alymphoid (they lack T, B as well as NK cells). After 10 days spleen cells were analyzed by FACS. Normal ratios of CD4 to CD8 T cells were found in reconstituted mice (Figure 4.3.4). The majority of them were blasting, indicating active proliferation. LAG-3 was clearly detectable on about 50% of blasts (Figure 4.3.4). This data suggests a role for LAG-3 in homeostasis. In fact, the function of LAG-3 during T cell homeostasis was recently implied from the experiments involving LAG-3 deficient mice, but the surface expression of LAG-3 in wild type animals has not been demonstrated. Workman and Vignali reported that, CD4+ and CD8+ LAG-3 deficient T cells show enhanced homeostatic expansion in the spleen of lymphopenic host (2.6 fold), which was abrogated by ectopic expression of WT LAG-3 (Workman and Vignali, 2005). Based on the findings that the spleens of 16 weeks old LAG-3 ko mice not only have increased numbers of T cells (2 fold), but also of B cells, granulocytes, macrophages and DCs they go on to suggest that cells that express LAG-3 (that is T cells) regulate the expansion of cells that do not (other leukocyte subtypes). Of particular interest is the observation that there was a substantial increase in splenic pDCs (4 fold) in LAG-3 ko mice. In the light of the findings presented in this thesis, especially the high expression of LAG-3 on activated B cells and pDCs (see section 4.5), their data could be reinterpreted as indicating LAG-3 mediated regulation of B cell and DC homeostasis.

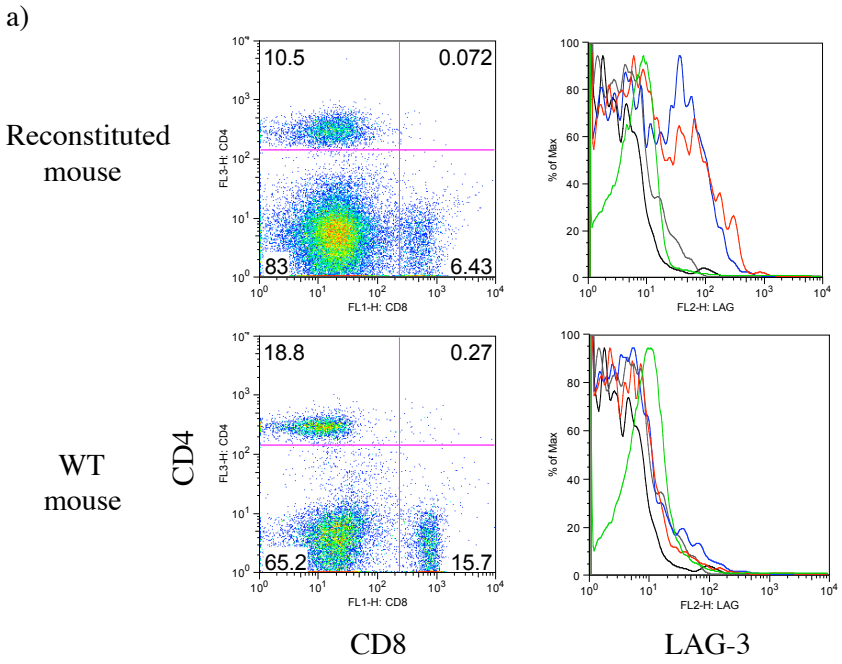


Figure 4.3.4. CD4 and CD8 T cells undergoing homeostatic expansion express LAG-3. LN cells from normal mice were transferred into a lymphopenic recipient. After 10 days, splenocytes of reconstituted and WT mice were analyzed for LAG-3 expression. Analysis of large blasts is presented. CD4+ and CD8+ populations are shown in left side panels. LAG-3 staining of CD8 gated cells is shown in red (secondary reagent control in grey), of CD4 gated cells in blue (secondary reagent control in black) and of CD8-CD4- cells in green (right side panels). The small cells do not express LAG-3 (not shown). The data are the representative of two independent experiments.

4.3.2. Is LAG-3 required for T reg suppressive activity?

The experiments involving ectopic expression of LAG-3 on A5 cells (section 4.1) suggested that this molecule has an immunoregulatory function on T cells, in agreement with reports by others (Hannier et al., 1998; Huard et al., 1996; Workman et al., 2002a); LAG-3 can act as a negative regulator of T cell activation. Interestingly, it was recently proposed that LAG-3 controls T cell function not only intrinsically (on the surface of effector T cells) but also extrinsically by its presence on regulatory T cells. In the recent report Huang and colleagues suggested that LAG-3 is important for the suppressive function of regulatory T cell subpopulation (Huang et al., 2004).

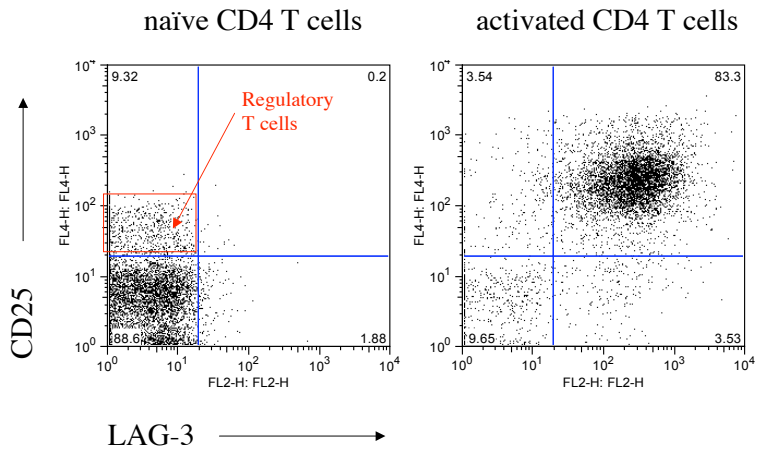


Figure 4.3.5. Non-activated regulatory T cells do not express LAG-3 on their surface. Freshly isolated or αCD3 and αCD28 activated (2 days) splenocytes from WT mice were stained for CD4, CD25 and LAG-3. The analysis was done on CD4 gated cells. The CD25+ population representing about 9% of total CD4 population (left panel) is LAG-3 negative.

Figure 4.3.5 shows that the CD25+ CD4+ T reg cells freshly isolated from a mouse are LAG-3 negative. In contrast, most of CD4 T cells activated with anti-CD3 and anti-CD28 Abs for two days stain positively for LAG-3. However, at this stage all activated cells express CD25 and T regs cannot be distinguished anymore. To verify LAG-3 expression on T regs, the experiment was repeated with purified naïve/effector (CD4+CD25-) and regulatory (CD4+CD25+) T cell populations. The purified cells were additionally labelled with CFSE allowing for easy detection of their proliferation.

Mouse regulatory T cells when stimulated and cultured alone show anergic phenotype, they proliferate poorly after TCR stimulation, when compared to effector T cells (Figure 4.3.6 a and b). This is the consequence of their dependence on exogenous IL-2, to such a degree that CD4+ CD25+ T cells are absent from IL-2 deficient mice (Papiernik et al., 1998). Yet, the regulatory cells that managed to proliferate up-regulated surface LAG-3, just like the effector population. It could be argued that this population resulted from contaminating effector T cells, unfortunately without a specific marker for T regs available, it is impossible to rigorously examine it. However, when T regs were mixed with non-labelled effector cells in 1 to 4 ratio and stimulated via their TCR, they proliferated just as well as effector cells alone and expressed the same levels of surface LAG-3 (Figure 4.3.6 c).

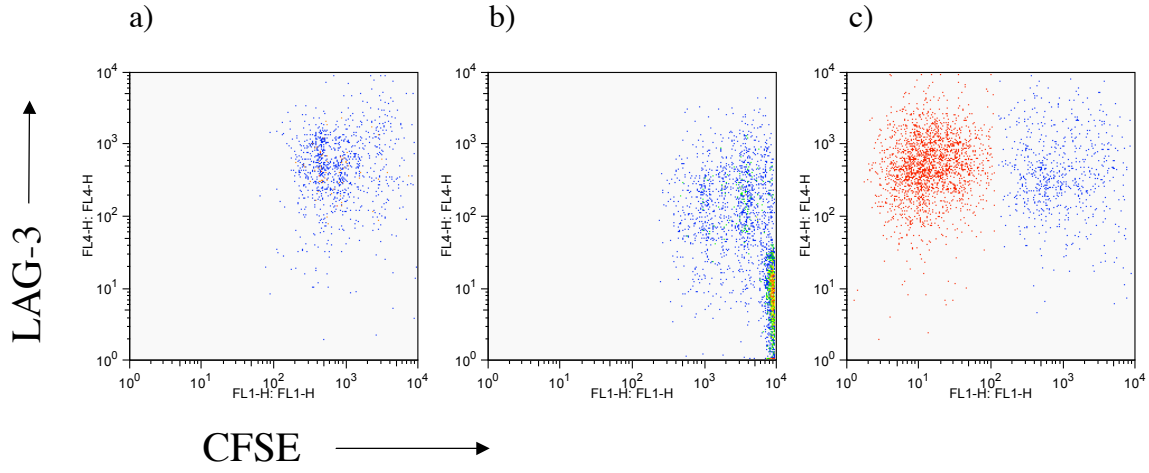


Figure 4.3.6. LAG-3 expression on regulatory versus effector T cells. The purified LN and spleen (a) effector (CD4+CD25-) and (b) regulatory (CD4+ CD25+) T cell populations were CFSE labeled and independently stimulated with soluble αCD3. After 4 days LAG-3 surface staining was performed. (c) CFSE labeled regulatory T cells (blue) were mixed with non-labeled effector T cells (red) in 1 to 4 ratio, respectively, and stimulated with soluble αCD3 for four days. LAG-3 staining and T regs CFSE dilution profile are shown.

In conclusion, consistent with the results of Huang et al., we could show that natural CD4+ CD25+ T regs express LAG-3 upon activation, which is significantly enhanced in the presence of effector cells. Naïve CD4+ CD25+ regulatory cells have no surface LAG-3, although reportedly they show significantly higher levels of LAG-3 mRNA (Huang et al., 2004). This discrepancy could be explained by the possibility of intracellular LAG-3 storage. Furthermore, after activation (monitored by CFSE dilution) the level of LAG-3 on T regs is the same as on classical effector cells, suggesting that LAG-3 cannot be used as a marker to distinguish T reg population.

To assess whether LAG-3 is indispensable for the suppressive function of regulatory T cells, we subjected WT as well as LAG-3 deficient T cells to the standard *in vitro* suppressor assay. The CFSE labelled WT effector T cells were cultured alone or were mixed in 1 to 1 ratio with WT or LAG-3-deficient unlabeled regulatory T cells, and the proliferation of effector cells was followed by CFSE dilution. As shown in Figure 4.3.7, the WT and LAG-3 deficient T regs were able to suppress the proliferation of WT effector T cells to the same extent suggesting that LAG-3 is dispensable for suppressor activity of T regs. The suppressive effect of WT and LAG-3 KO T regs was observed

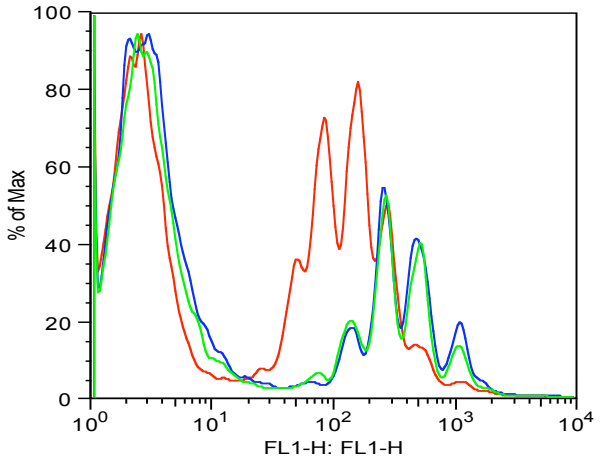


Figure 4.3.7. The deletion of LAG-3 does not affect the suppressive activity of T regs. CFSE labeled WT effector cells were mixed with unlabeled WT effector cells (in red) as controls, WT Tregs (in blue) or LAG-3 deficient Tregs (in green) in 1 to 1 ratio. The cells were stimulated with 0.1ug/ml of anti-CD3 in the presence of irradiated APCs for 4 days. The effector cell proliferation was followed by CFSE dilution.

over a range of anti-CD3 concentrations (0.1 ug/ml to 5 ug/ml) and was independent of LAG-3 expression on T cells as also LAG-3 deficient effector cells could be suppressed by both T reg variants (not shown). Furthermore, the addition of monoclonal anti-LAG-3 antibodies to WT T regs also did not affect their activity (not shown).

The above results are clearly in disagreement with the report by Huang and colleagues (Huang et al., 2004). This discrepancy can be partially explained by different assays and models used. Huang et al. used a very specific *in vivo* model and non-standard *in vitro* assays. Also they do not clearly define 'regulatory ' population and the effects they observed were generally quite weak.

Our results do not support the notion that LAG-3 is a specific marker for T regs. Any type of *in vitro* activated T cell becomes strongly LAG-3 positive.

4.4. T-cell induced expression of LAG-3 on B cells

'Expression of lymphocyte activation gene 3 (LAG-3) on B cells is induced by T cells' Kisielow M., Kisielow J., Capoferri-Sollami G. and Karjalainen K. 2005. *Eur.J. Immunol. 35: 2081-2088.*

European Journal of Immunology

Expression of lymphocyte activation gene 3 (LAG-3) on B cells is induced by T cells

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Lymphocyte activation gene 3 (LAG-3/CD223) is a CD4 homolog known to be selectively expressed in activated T and NK cells. It is thought to have a negative regulatory function in T cells. With the help of new monoclonal antibodies against mouse LAG-3, we show that LAG-3 surface expression is not limited to activated T and NK cells but is also found on activated B cells. Induction of B cell surface expression is T cell dependent and mediated by a soluble factor. The majority of LAG-3 on B cell surface is endogenously produced, even though soluble LAG-3 is present in the culture supernatants and can be passively absorbed. As B cells express LAG-3 in a T cell dependent manner and not when activated by Toll-like-receptor agonists alone, we propose LAG-3 as a new marker of T cell induced B cell activation.

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Key words: B cells · T cells · Cell activation · Cell surface molecules

Introduction

Lymphocyte activation gene 3 (LAG-3/CD223) was first identified as a cell surface molecule selectively expressed in activated human NK and T lymphocytes [1, 2]. Interestingly, it was found to be closely related to CD4 [1]. Like CD4, LAG-3 binds to MHC class II molecules on the surface of APC [2]; however, LAG-3 does it with much higher avidity, suggesting that it might act as a natural competitor of CD4. This notion was supported by experiments showing that LAG-3 can interfere with interactions between MHC class II and CD4 [3–6].

The mouse homologue of LAG-3 shows 69.9% protein sequence identity to the human protein. All of the putative structurally important residues are well conserved, thus predicting the same folding pattern [7]. Murine LAG-3 transcripts were found in the spleen, thymus and brain. A very small percentage of αβ T and NK cells in naive mice express LAG-3, whereas a significant percentage of γδ T cells show LAG-3 expression [8].

The initial analysis of LAG $3^{-/-}$ mice did not reveal any defect in T or B cell function [9]. However, more recent experiments showed that murine LAG-3 acts as a negative regulator of T cell function *in vitro* [10] and as an inhibitor of antigen-driven T cell expansion *in vivo* [11], in agreement with earlier studies on human LAG-3 [4, 6]. Furthermore, a role of LAG-3 in regulatory T cells has been suggested [12]. It was also suggested that LAG-3 may define different modes of NK killing [9], but this notion was not supported by studies with human NK cells [13].

Several alternative mRNA splice-variants of human LAG-3 have been described, two of them encoding potential secreted forms: LAG-3V1 (*i.e.* the D1–D2 domains of the protein, 36 kDa) and LAG-3V3 (D1–D3, 52 kDa) [14]. The longer form was detected by ELISA in the serum of healthy individuals as well as of tuberculosis patients with a favorable outcome [15–16]; in addition, LAG-3 expression by T cell clones correlated with IFN-γ production, and hence soluble LAG-3 has been suggested as a serological marker of Th1 responses [17]. In addition, LAG-3 was identified in the supernatants of LAG-3-expressing T cell hybridoma cultures and was detected in the serum of WT mice [18].

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Abbreviations: **ko:** Knockout · **LAG-3:** Lymphocyte activation gene 3 · **SAS:** Saturated ammonium sulfate

However, LAG-3 appears to have a more complex role in the immune system than just being a regulator of T cell responses. Different functions may be attributed to its soluble and membrane-bound forms. A soluble murine LAG-3–Ig fusion protein induced MHC class II mediated signaling in DC, leading to their activation and/or maturation as well as production of cytokines [19–22]. In addition, mLAG-3–Ig was shown to reduce tumor growth [23, 24] and to increase CD8 cytotoxic responses and CD4 Th1 responses in mice [25].

Because of its relation to CD4 and its binding to MHC class II, studies on LAG-3 have concentrated on its role in T cell function. In this study, we use new mAb against mouse LAG-3 to show that this molecule is also expressed on activated B cells. LAG-3 expression on B cells is T cell induced and is mediated by an unknown soluble factor. These observations shed a new light on the role of LAG-3 in the immune system and identify LAG-3 as a potential novel marker of T cell induced B cell activation.

Results and Discussion

Generation and characterization of mAb against mouse LAG-3

In order to study the expression and function of LAG-3, we produced a number of new mAb against mouse LAG-3. As shown in Fig. 1A, the G15, G37 and G40 Ab, as representatives of a large panel (at least 6 different epitopes are recognized by 12 mAb), stain activated T cells from normal but not from LAG-3 knockout (ko) mice. In activated splenocyte cultures, LAG-3 was up-regulated on day 1 and reached plateau on day 3 of activation, consistent with previous observations [8]. In addition, LAG-3 expression was consistently 2.0–2.5-fold higher on $CD8^+$ than $CD4^+$ T cells (Fig. 1B), in agreement with studies on human T cells [26].

We confirmed that LAG-3 can down-modulate T cell activation by using $LAG\text{-}3^+$ and $LAG\text{-}3^-$ variants of the A5 T cell hybridoma as antigen-specific responders. We used this assay to show that our mAb were functionally active. As shown Fig. 1C, mAb G15 and G37 very efficiently neutralize the inhibition of T cell activation caused by LAG-3 surface expression. G40 is less efficient, which may be due to the fact that the epitope it recognizes (*i.e.* the D3–D4 part of the protein) is closer to the membrane than the epitopes of G15 and G37 (recognizing the D1–D2 fragment) are. The LAG-3 mAb had no effect on $LAG\text{-}3^-$ A5 cells (not shown).

Altogether these experiments show that the new mAb are LAG-3-specific and can neutralize LAG-3 function in T cells.

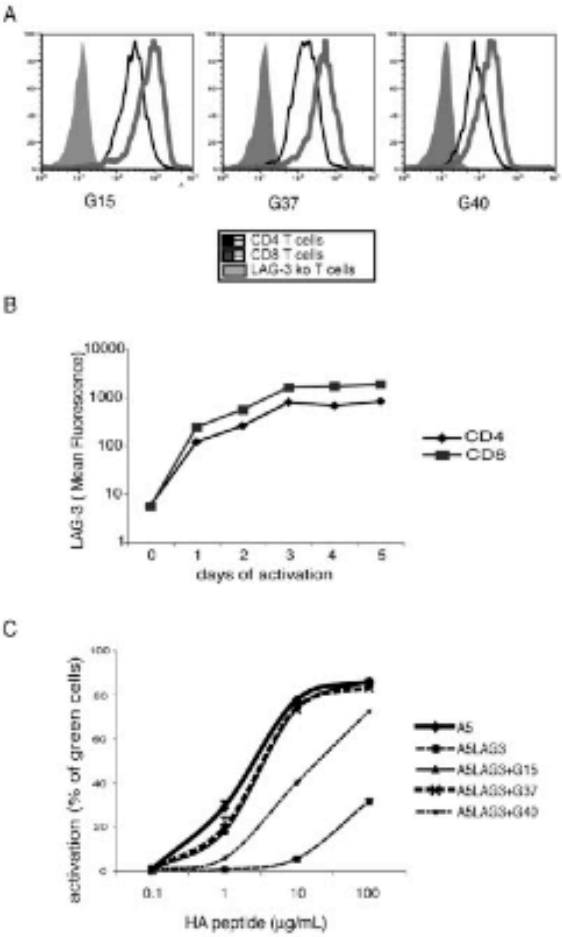

Fig. 1. Staining of activated T cells and functional activity of mAb that recognize mouse LAG-3. (A) WT or LAG-3 ko splenocytes were plated on plates coated with anti-CD3 plus anti-CD28 and were stained with different anti-LAG-3 antibodies on day 4 of activation. Gray line, CD8 T cells; black line, CD4 T cells; shaded area, LAG-3 ko T cells. (B) A time-course analysis was performed. The data represent at least four independent experiments. (C) A20 cells were loaded with HA peptide at the indicated concentrations for 16 h in the presence or absence of various mAb and used to activate A5 or A5LAG-3 cells carrying an NFAT-GFP readout transgene. The graph shows the average of at least two separate experiments done in duplicate.

Activated B cells express LAG-3

In our activation assays of splenocytes we observed that, although on day 1 the cells expressing LAG-3 were mostly T cells, on day 3 of culture all cells stained for LAG-3, including the bystander-activated $CD19^+$ cells (Fig. 2A). The LAG-3 surface levels on B cells were consistently 4–6-fold lower than on T cells and proportional to the level of T cell activation. The

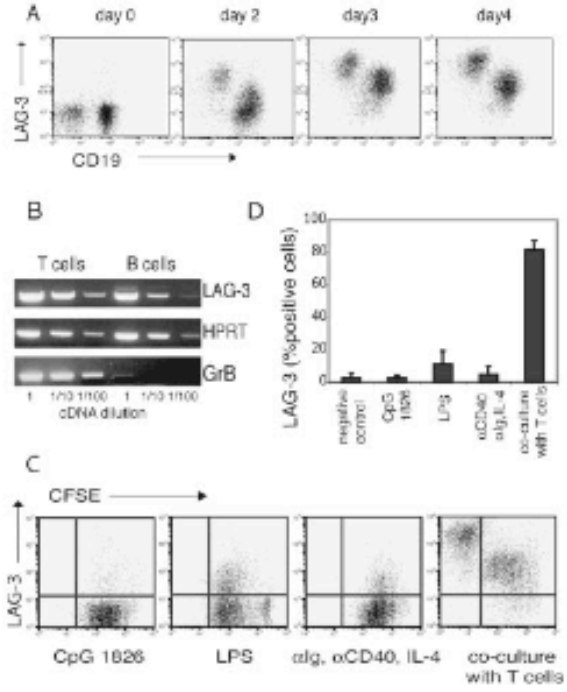

Fig. 2. B cells show pronounced levels of surface LAG-3 only in the presence of activated T cells. WT splenocytes were stimulated on immobilized anti-CD3 and analyzed for LAG-3 expression at different time points. (A) The CD19/LAG-3 double-staining represents at least four independent experiments. $CD19^-$ cells are mainly T cells. (B) LAG-3 RT-PCR was performed on RNA samples from T or B cells from splenocytes activated for 4 days. Granzyme B (GrB) primers were used to check the purity of the B cell sample. (C) Purified B cells were CFSE-labeled and cultured in the normal growth medium with various stimuli: CpG 1826; LPS; anti-Ig plus anti-CD40 plus IL-4 (αCD40, αIg, IL-4); or anti-CD3-activated unlabeled T cells. B cells were analyzed for LAG-3 expression versus CFSE after 4 days of culture. The cells staining strongly for LAG-3 in the upper left quadrant of the rightmost panel are T cells. (D) The average of the percentage $LAG\text{-}3^+$ B cells from two (CpG 1826) or four different experiments.

expression of LAG-3 by B cells was also confirmed by RT-PCR on purified cell populations (Fig. 2B). The B cell population showed barely detectable contamination of T cells, as determined by Granzyme-B-specific RT-PCR (Fig. 2B).

The above results show that LAG-3 expression is not limited to activated T and NK cells, but is also up-regulated in activated B cells. To our knowledge, none of the previous studies observed this. However, in mice the analysis was limited to the A20 B cell lymphoma, where no expression was detected by Northern blots [7]. Similarly, a number of transformed human B cell lines (LAZ 388, LAZ 461, RAJI, RAMOS, DAUDI and E418) were found to be $LAG\text{-}3^-$ [1, 2]. LAG-3 was not found on resting or cultured (PWM-stimulated) human peripheral blood B cells expressing the CD20 molecule or in tonsils [2]. We found that activated T cells are indispensable for LAG-3 up-regulation on B cells (see below), which could explain the negative results in the above-mentioned studies.

LAG-3 expression on B cells is T cell dependent

Since LAG-3 expression in B cells was rather unexpected, we set out to further characterize that observation. Purified $CD19^+$ cells from WT spleens were CFSE-labeled and incubated with various stimuli: the unmethylated CpG motif 1826; bacterial LPS; anti-Ig antibody in combination with anti-CD40 and IL-4; or only with anti-CD3-activated T cells. CpG and LPS are both inducers of innate immunity: CpG 1826 activates B cells through TLR9; and LPS signals through TLR4. Anti-Ig antibody, on the other hand, cross-links membrane Ig and anti-CD40 antibody with IL-4 mimic T cell help.

As shown in Fig. 2C, although B cells were proliferating in response to all stimuli, as visualized by CFSE dilution, only co-culture with activated T cells led to pronounced surface expression of LAG-3 (Fig. 2C and D). Stimulation of B cells with LPS consistently showed a staining 'tail', possibly representing a sub-population of B cells. However the percentage of $LAG\text{-}3^+$ cells after LPS activation was always low and showed the greatest variation. Surprisingly, BCR triggering by anti-Ig antibody plus T cell help imitated by co-stimulation with anti-CD40 and IL-4 did not induce LAG-3 expression. This observation indicates a more complex interaction between T and B cells and suggests caution when using anti-CD40 and IL-4 as a substitute for T cell help.

Currently, LAG-3 is considered to be a marker of activated T cells (both CD4 and CD8) and NK cells, and it was suggested that it might be more optimal than CD69, because it is not expressed by B cells [8]. We demonstrate here that the situation is more complicated. Actually, LAG-3 may prove to be a marker of T cell mediated B cell activation (see below). It is, however, not an early marker. It shows delayed kinetics as compared with common activation markers CD69 or CD25, likely resulting from the requirement for T cells to be activated first. Kinetically it more resembles the up-regulation of CD80 (M. K., unpublished observation).

The majority of LAG-3 on B cells is endogenously produced

Although RT-PCR analysis showed that B cells can produce their own Lag-3 mRNA, we wanted to exclude the possibility that LAG-3 on the surface of B cells was mainly due to passive absorption. In order to investigate

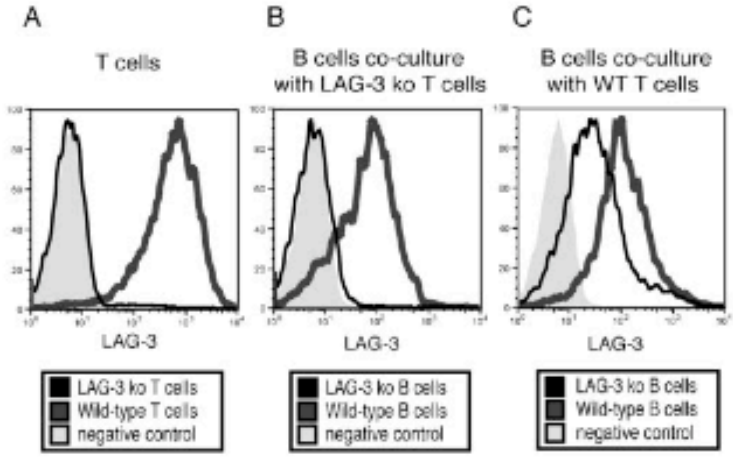

Fig. 3. B cells produce most of their surface LAG-3. The LAG-3 surface staining of WT or LAG-3 ko lymphocyte co-cultures after 4 days of anti-CD3 plus anti-CD28 activation is shown. Purified T cells and B cells from WT or LAG-3 ko mice were mixed at a 1:1 ratio in various combinations: (A) WT (thick line) or LAG-3 ko (thin line) T cells co-cultured with WT B cells; (B) WT (thick line) or LAG-3 ko (thin line) B cells co-cultured with LAG-3 ko T cells; (C) WT (thick line) or LAG-3 ko (thin line) B cells co-cultured with WT T cells.

that, we purified T or B cells from spleens of C57BL6 WT and LAG-3 ko mice [9], mixed them in various combinations and cultured in plates coated with anti-CD3 plus anti-CD28. On day 4 of culture the samples were stained for surface LAG-3. As expected, WT T cells were brightly positive, whereas LAG-3 ko T cells were not (Fig. 3A). Importantly, WT B cells, but not LAG-3 ko B cells, became strongly positive in co-cultures with LAG-3 ko T cells, clearly demonstrating the endogenous production of LAG-3 by B cells (Fig. 3B). In contrast, LAG-3 ko B cells became only weakly positive (25% of the level of WT B cells) in co-cultures with WT T cells, indicating that some absorption can occur (Fig. 3C).

Since LAG-3 was shown to be an MHC class II ligand [2, 8] and since activated B cells up-regulate MHC class II on their surface, this absorption could be mediated by MHC class II. To test the importance of MHC in passive absorption of LAG-3, we co-cultured double-ko B cells (LAG-3 ko plus MHC class II ko) with WT T cells (or total splenocytes) on plates coated with anti-CD3 plus anti-CD28. Both LAG-3 ko and LAG-3 / MHC class II double-ko B cells showed equal (similar to Fig. 3B) levels of LAG-3 on the surface, indicating no role for MHC class II in LAG-3 absorption (data not shown). These data suggest the existence of an additional LAG-3 ligand. This ligand could bind soluble LAG-3 or could be involved in LAG-3 "pick-up" directly from the surface of T cells in a similar manner to the acquisition by T cells of B7 molecules from APC [27].

Soluble LAG-3 is detectable in splenic cultures

Since we could detect some passive acquisition of LAG-3 by B cells, we analyzed whether soluble LAG-3 can be found in cultures of stimulated T cells. Indeed, we could easily demonstrate the presence of soluble LAG-3 in our activated splenic cultures (Fig. 4). Western-blot analyses revealed a soluble LAG-3 protein with an apparent molecular weight of 53 kDa that was not present in cultures derived from LAG-3 deficient mice. This protein exhibited similar mobility to our soluble recombinant LAG-3 composed of all four extracellular domains, but different from the membrane-bound LAG-3 that migrates at approximately 75 kDa. The 53-kDa protein might be a mouse homologue of the predicted human LAG-3V3 (D1–D3, soluble) variant resulting from alternative splicing [14, 15]. Also, soluble LAG-3, with a predicted size of 54 kDa, was recently isolated from an activated LAG-3-transduced T cell hybridoma. Interestingly, it was shown to be the result of proteolytic processing rather than differential splicing [18]. Further studies are required to determine the exact composition of the soluble protein. We could not detect any other species of soluble LAG-3, especially the one correspond-

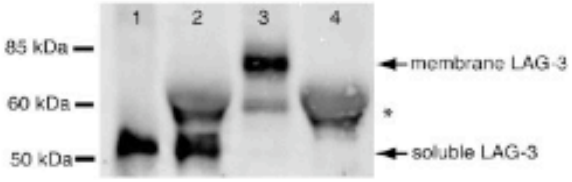

Fig. 4. Soluble LAG-3 is detectable in activated splenocyte cultures. Various samples were subjected to 7.5% PAGE under reducing conditions: lane 1, recombinant soluble LAG-3 (D1–D4); lane 2, SAS precipitate from the supernatant of WT splenocytes cultured for 4 days in anti-CD3 plus anti-CD28; lane 3, cell lysate of CD8 cells purified from WT splenocytes cultured for 4 days in anti-CD3; lane 4, SAS precipitate of the supernatant of LAG3 ko splenocytes cultured for 4 days in anti-CD3 plus anti-CD28. The blot was probed with anti-LAG-3 polyclonal Ab. SAS precipitates of WT and LAG-3 ko culture supernatants from three independent experiments were analyzed. * non-specific background.

ing to potential human LAG-3V1 with predicted molecular weight of 36 kDa [14].

LAG-3 expression on B cells is T cell induced and mediated by a soluble factor

To further characterize the requirements for the endogenous expression of B cell LAG-3, we tested whether contact between T and B cells was necessary. We first performed Transwell experiments in which we placed CFSE-labeled B cells in the upper compartments and total splenocytes in the anti-CD3-coated lower compartments. The 0.4-μm membrane separating the compartments allows the passage of small molecules but not cells. After 4 days of activation, we looked at the LAG-3 levels on B cells in the lower and upper compartments as well as B cell proliferation in the upper compartments. In the normal growth medium, we observed that although T and B cells were activated in the lower compartment, hardly any B cells proliferated and expressed LAG-3 in the upper compartment (Fig. 5A). This observation shows that the transmigrating cytokines are not enough to induce LAG-3 expression on B cells. However, when we stimulated B cells by adding anti-Ig, anti-CD40 and IL-4 to the culture medium, proliferating B cells in the upper compartment became LAG-3$^+$ (Fig. 5B). After three or four divisions the majority of B cells were LAG-3$^+$.

Furthermore, when B cells were stimulated, in a similar Transwell experiment, with CpG 1826 or LPS in the presence of activated T cells some of them (24% and 17%, respectively) stained positively for LAG-3 (data not shown). This observation suggests that once B cells are stimulated to proliferate in the presence of activated T cells, they can express LAG-3 regardless of the type of trigger. However, in "the innate scenario" it is unlikely that B cells become positive *in vivo* due to more-delayed kinetics of T cell activation.

The above data strongly suggested that a soluble factor might be involved in the process. We then checked whether the potential LAG-3-inducing factor could be transferred with culture supernatant. Indeed, LAG-3 was up-regulated on purified WT B cells that were stimulated with anti-Ig, anti-CD40 and IL-4 during culture in growth medium containing supernatants from splenocytes cultured for 4 days on anti-CD3 plus anti-CD28; B cells treated with the same proliferation stimuli but cultured in normal growth medium did not up-regulate LAG-3 on their surface (Fig. 5C). In addition, the lack of LAG-3 staining on LAG-3 ko B cells strengthens the point that LAG-3 on B cells is endogenously produced (Fig. 5C, graph).

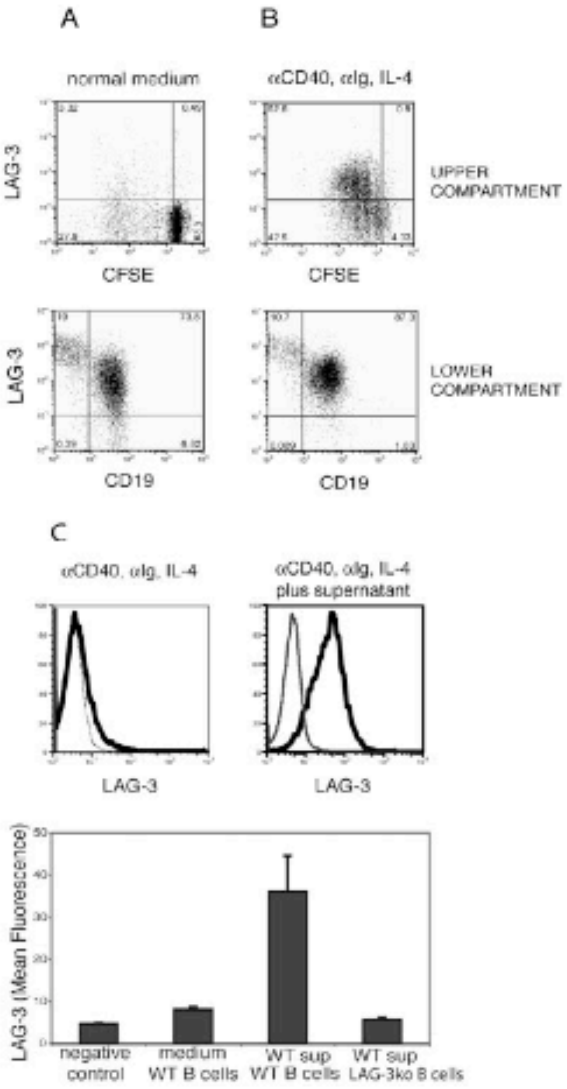

Fig. 5. LAG-3 expression on B cells is mediated by a soluble factor. WT splenocytes were added to anti-CD3-coated lower compartments of a Transwell, while purified CD19$^+$, CFSE-labeled cells were put in the upper compartments. Cells were cultured in the absence (A) or presence (B) of an anti-Ig, anti-CD40 and IL-4 (αCD40 αIg, IL-4) cocktail in normal growth medium for 4 days, followed by LAG-3 staining. One representative experiment of four is shown. (C) Purified B cells (CD19$^+$) were cultured in the presence of anti-Ig, anti-CD40 and IL-4 in normal growth medium (left panel) or in supernatants from splenocytes cultured for 4 days on anti-CD3 plus anti-CD28 (right panel). Supernatants were carefully collected, centrifuged (450×g, 10 min) and filtered through a 0.2-mm filter. Thick black line, LAG-3; thin grey line, negative control. The average data of four different experiments performed with WT B cells or LAG-3 ko B cells are shown (bar graph).

Till now we have failed to identify the soluble LAG-3-inducing molecule. We have tested a number of cytokines produced by activated T cells, namely IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, TNF-β and GM-CSF, by adding them to sorted B cells stimulated to proliferate in the normal growth medium supplied with a given cytokine at increasing concentrations. Only addition of IL-6 had an effect, resulting in a low fraction (5–15%) of LAG-3$^+$ cells. We also tested BAFF, since it is an important B cell survival factor, but this molecule also failed to induce LAG-3 on B cells (data not shown).

So far, analysis of LAG-3 function has concentrated on T cell responses [4, 6, 10, 12, 28]. The identification of LAG-3 on the surface of activated B cells adds a new perspective to LAG-3 function in the immune system and may redirect future LAG-3 studies. The reported LAG-3 binding to MHC class II may not be the only mode of LAG-3 action. The fact that MHC-class-II$^-$ B cells can still bind LAG-3 suggests the existence of an additional LAG-3 ligand. Interaction with this ligand could be the reason for higher levels of LAG-3 on CD8 than on CD4 T cells ([26], this report); alternatively CD8 T cells could use LAG-3 to activate MHC-class-II$^+$ cells, for example DC.

In addition, we would like to propose LAG-3 as a novel marker of T cell induced B cell activation, even though its function on B cells remains to be determined. Conceivably, it could play a role in the antigen presentation by B cells. The experiments to test this hypothesis are under way.

Materials and methods

Cell culture

For all mouse cell culture experiments, normal growth medium, *i.e.* IMDM supplemented with 2% FCS (Seromed) and 0.03% PRIMATONE® RL/LF (Quest International), was used.

Production of anti-mouse-LAG-3 mAb

Lewis rats were immunized with LAG-3-transduced rat cells. Specific hybridomas were selected by FACS and mAb were partially epitope-mapped using recombinant soluble LAG-3 molecules consisting of either the D1–D2 or D1–D4 domains (not shown). Ten Ab were purified (Sepharose G+ beads, Pharmacia), biotinylated (Molecular Probes) and their specificity was ascertained by their ability to stain splenocytes derived from WT mice, but not from LAG-3 ko mice, after activating the splenocytes with anti-CD3 and anti-CD28. The Ab were isotyped by ELISA using goat Ab specific for rat isotypes (Pharmingen). Ab G15, G37 and G40 are all of the IgG2a isotype.

Mice

C57BL6 (WT) mice were purchased from Harlan Italy (San Pietro al Natisone, Italy). LAG-3-deficient (LAG-3 ko) mice were provided by Dr D. A. Vignali (Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN, USA) with permission from Dr Diane Mathis (Joslin Diabetes Center, Boston, MA, USA). Animals used were 6–12 weeks old and age- and sex-matched.

Culture and activation of A5 and A5LAG-3 cells

A5 is an HA-peptide-specific T cell hybridoma carrying GFP under the control of triple NFAT binding sites [29]. A5LAG-3 cells are A5 cells stably transfected with a plasmid harboring the LAG-3 cDNA sequence under the control of the EF-1a promoter. A20 B lymphoma cells were used as APC. A20 cells were γ-irradiated (30 Gy) and then loaded with various concentrations of HA peptide for 2 h. Anti-LAG-3 Ab were added to A20/A5LAG-3 cultures at 10 μg/ml. The activation state of A5 and A5LAG-3 cells was assessed by determining the percentage of green cells after 16 h (FACS Calibur).

Culturing and activation of splenocytes

Mice were sacrificed by CO_2 inhalation, and suspensions of spleen cells were prepared in normal growth medium. Red blood cells were depleted using Gey's solution. For activation, 24-well plates were coated with anti-CD3 (145.2C11) and anti-CD28 (37.51) Ab (BD Pharmingen) at 2 μg/ml.

Flow cytometry and cell sorting

All stainings were performed with the pre-treatment and in the presence of anti-mouse-CD16/CD32 (2.4G2) Ab to block FcR. For staining, anti-CD4–PE or –PercP (L3T4), anti-CD8–allophycocyanin or –PercP (53-6.7) and anti-CD19–FITC (ID3) (BD Pharmingen) were used.

Biotinylated mAb G15 or G37 and G40 were used for LAG-3 surface staining with streptavidin–PE or streptavidin–allophycocyanin as a secondary reagent. Cell sorting was performed with BD FACSAria.

RT-PCR

Splenocyte cultures were collected on indicated days and stained as described above.

CD4$^+$, CD8$^+$ or CD19$^+$ cells were purified by cell sorting and lysed with RNAzol B (TEL-TEST, Inc.) according to the manufacturer's instructions. First-strand cDNA synthesis was performed according to a standard protocol. LAG-3 primers encompassing exons 5–8 were used for Lag-3 amplification: 5'TCATTGCCAAGTGGACTCC, 3'TCAGAGCTGCCTGGGCTCTGG. HPRT primers used for cDNA normalization were 5'GCTGGTGAAAAGGACCTCT, 3'CACAGGACTAGAACACCTG. Granzyme-B-specific primers were 5'CAGGAGATGTGTGCTATGTGG, 3'TAGAGCAATCCTGGACTCAGC.

B cell purification, CFSE labeling and stimulation

B cells were sorted as described above or MACS purified (freshly isolated splenocytes were labeled with biotinylated anti-CD19 Ab (ID3, BD Pharmingen), followed by incubation with streptavidin MicroBeads and separated on the MS separation columns (Miltenyi Biotec) according to the manufacturer's instructions). Purified B cells were suspended at 10^7 cells/ml in PBS plus 0.1% BSA and incubated with CFSE at a final concentration of 5 μM (Molecular Probes) for 10 min at 37°C. Labeling was quenched by addition of a 10× volume of PBS plus 0.1% BSA. Cell were washed, resuspended in culture medium, plated in 96-well plates (1×10^5 cells/well) and stimulated with CpG 1826 at final concentration of 0.3 μg/ml [30], with LPS at final concentration of 25 μg/ml (L-2630 from Sigma), or with purified anti-mouse-CD40 mAb (FGK-45) and anti-mouse-Ig (κ light-chain) mAb (187.1) (10 μg/ ml each) supplemented with IL-4 (from the culture supernatant of the IL-4-producing X63 cell line). IL-5, IL-10, IL-12, IL-13, IFN-γ and TNF-β were used at 20–30 ng/ml as well as 5× and 10× concentrations. For stimulation with IL-2, IL-4, IL-6 or GM-CSF, media conditioned with the respective cytokines were used. Recombinant BAFF (a kind gift from M. Rauch, DKBW, Basel) was used at concentrations from 32.0 ng/ml to 2.6 μg/ml. Control B cell survival assays showed BAFF activity at 80 ng/ml.

SAS precipitation and Western blot

Splenocyte culture supernatants were centrifuged 30 min at 20,000×g at RT and transferred to fresh tubes. Saturated ammonium sulfate (SAS) was added slowly with stirring up to 45% (v/v). Precipitation was carried out at 4°C for 2 h. Samples were centrifuged again for 30 min at 20,000×g at RT. Precipitates were dissolved in PBS. $CD8^+$ cells were sorted from anti-CD3-stimulated splenocytes on day 4 of activation and lysed in 1% Triton X-100, 150 mM NaCl, 50 mM Tris pH 8.3 with a Complete® protease-inhibitor cocktail (Roche). Samples were separated under reducing conditions and transferred onto nitrocellulose mambranes (Schleicher and Schuell), blocked with 4% blotting-grade milk (Bio Rad) in PBS and probed with anti-mouse-LAG-3 polyclonal rabbit Ab raised against a soluble mLAG-3–Ig fusion protein, followed by HRP-linked goat anti-rabbit-Ig (Southern Biotechnology) for detection.

Transwell experiments

Transwell cell culture chambers (6.5-mm diameter, 0.4-μm pore size) were purchased from Costar. The lower compartment was coated with purified anti-CD3 (145.2C11) Ab at 2 μg/ml. Splenocytes (2×10^6) were put into the lower compartment, CFSE-labeled $CD19^+$ cells (2×10^5) were placed in the upper compartment. The cells were cultured in normal growth medium alone or supplemented with purified anti-mouse-CD40 mAb (FGK-45) and anti-mouse-Ig (κ light-chain) mAb (187.1) (10 μg/ml each) supplemented with IL-4 (from the culture supernatant of the IL-4-producing X63 cell line). After two days of activation half of the medium was replaced by fresh medium.

Recombinant soluble LAG-3 production in *Drosophila* cells

Soluble LAG-3 was produced in *Drosophila* SL-2 cells as previously described [31]. The sequence encoding four extracellular domains (nucleotides 357–1769) was cloned into a pRmHa-3 vector by standard molecular techniques. Soluble LAG-3 was purified by affinity chromatography using the anti-LAG-3 mAb G40.

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4.4.1 Analysis of B cells - supplementary data

To complete the kinetic analysis of LAG-3 expression on B cells, we looked at the rate of down-regulation of LAG-3 on this lymphocyte subpopulation.

LAG-3 expression on the surface of B cells peaks on day 3 to 4 of culture with activated T cells and stays on at least till day 6. Purified B cells need both the proliferation stimulus (for example, provided by BCR triggering together with anti-CD40 help) and a soluble factor produced by T cells in order to express LAG-3 on their surface.

After withdrawal of proliferation stimuli LAG-3 disappears from the surface of B cells quickly, as demonstrated by the following experiment. B cells were purified by cell sorting from splenocyte cultures 3 days after stimulation with αCD3 and αCD28 Abs.

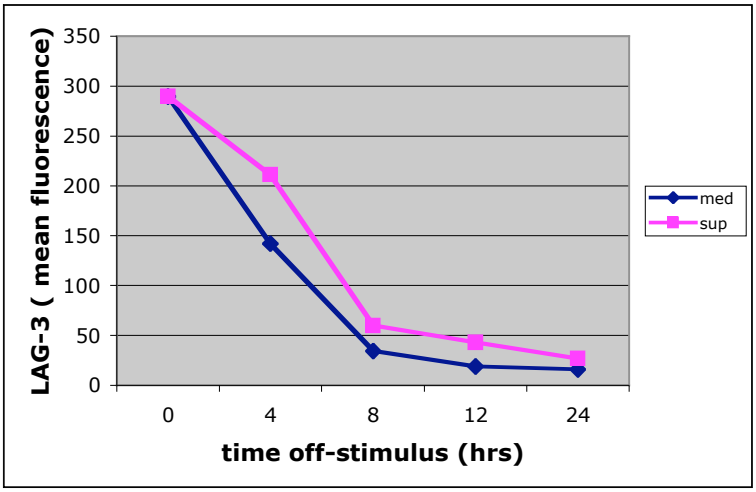


Figure 4.4.1. Down-regulation of surface LAG-3 on B cells after removal of proliferation stimuli. CD19 positive cells were sorted from splenocyte cultures activated for 3 days on immobilized αCD3 and αCD28 Abs, and resuspended in the normal growth medium (blue line) or in the day 3 activated splenocyte culture supernatant (pink line). LAG-3 levels were determined by surface staining with αLAG-3 mAb G15.

Purified B cells were then transferred to normal growth medium or kept in the supernatant collected from day 3 cultures from which B cells were sorted (containing the soluble factor(s) needed). LAG-3 surface expression of the purified B cells was followed over time.

Unlike T cells, which display high surface LAG-3 for at least 24 hrs after removal of proliferation stimulus (Figure 4.3.3), B cells loose surface LAG-3 within hours. After 4 hours 'off-stimulus' B cells show only 50% of the initial amounts of LAG-3 and after 8 hours almost all surface LAG-3 is gone (Figure 4.4.1). Thus the rate of LAG-3 down-regulation is much faster for B cells than for T cells, as T cells needed four days to reduce their LAG-3 surface expression by half. The soluble factor alone provided in the supernatant of activated splenocytes, that is responsible for up-regulation of LAG-3 on B cells, can delay surface LAG-3 down-regulation, but only to a small extent (about 30%). These results suggest that proliferation stimulus is required not only for up-regulation of LAG-3 on B cells, but also to keep LAG-3 surface levels up.

4.5. Characterization of LAG-3 expression on dendritic cells.

In order to comprehensively describe the expression pattern of LAG-3 in the immune system we also looked at various subsets of DCs.

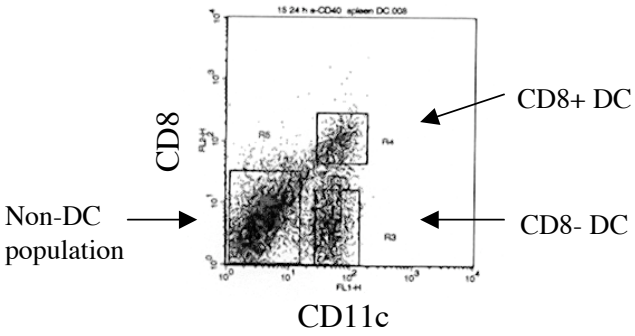
First, two DC subsets of the spleen; CD8+ ('lymphoid') and CD8- ('myeloid') cells were analyzed for LAG-3 expression. The isolated cells were cultured in the normal growth medium in the absence or presence of various stimuli: TNF-α, LPS, anti-CD40 Ab or combination of all three (mix). The two DC populations were defined using CD11c and CD8 as markers (Figure 4.5.1 a). CD11c/CD8/LAG-3 triple staining (Figure 4.5.1 b) showed that freshly isolated splenic CD8+ or CD8- DCs do not express LAG-3 on their surface. After 24 hours in culture without any stimulus, the subset of CD8- DCs up-regulated LAG-3 while CD8+ DC fraction did not. The LAG-3 expression on CD8- DC subset increased even more upon treatment with various stimuli, of which LPS was most effective. LAG-3 expression on CD8- without any stimulus is likely the consequence, of integrin-mediated DC maturation caused by adhesion of DCs to plastic.

Interestingly, none of the stimuli induced LAG-3 expression on CD8+ DCs and the majority of non-DC population remained negative as well. Staining of B7.2, a molecule known to be up-regulated after DC stimulation, confirmed that both DC subsets were activated (not shown).

The observation that LPS can induce expression of LAG-3 on CD8- DCs *in vitro* was supported by a set of *in vivo* experiments. C57BL6 mice were injected i.p. with LPS. This treatment was shown to induce DC maturation in the spleen (De Smedt et al., 1996). When both DC subsets, CD11c+CD8+ and CD11c+CD8-, were isolated from LPS treated mouse, only the CD8- fraction showed higher levels of LAG-3 compared to DC obtained from untreated animals (Figure 4.5.2).

CD8- DCs are widely distributed in both lymphoid and non-lymphoid tissues, while the CD8+ DCs are found in the lymph nodes and spleen, and in the thymus where they predominate (Steinman and Inaba, 1999; Vremec and Shortman, 1997). Considering the

a)



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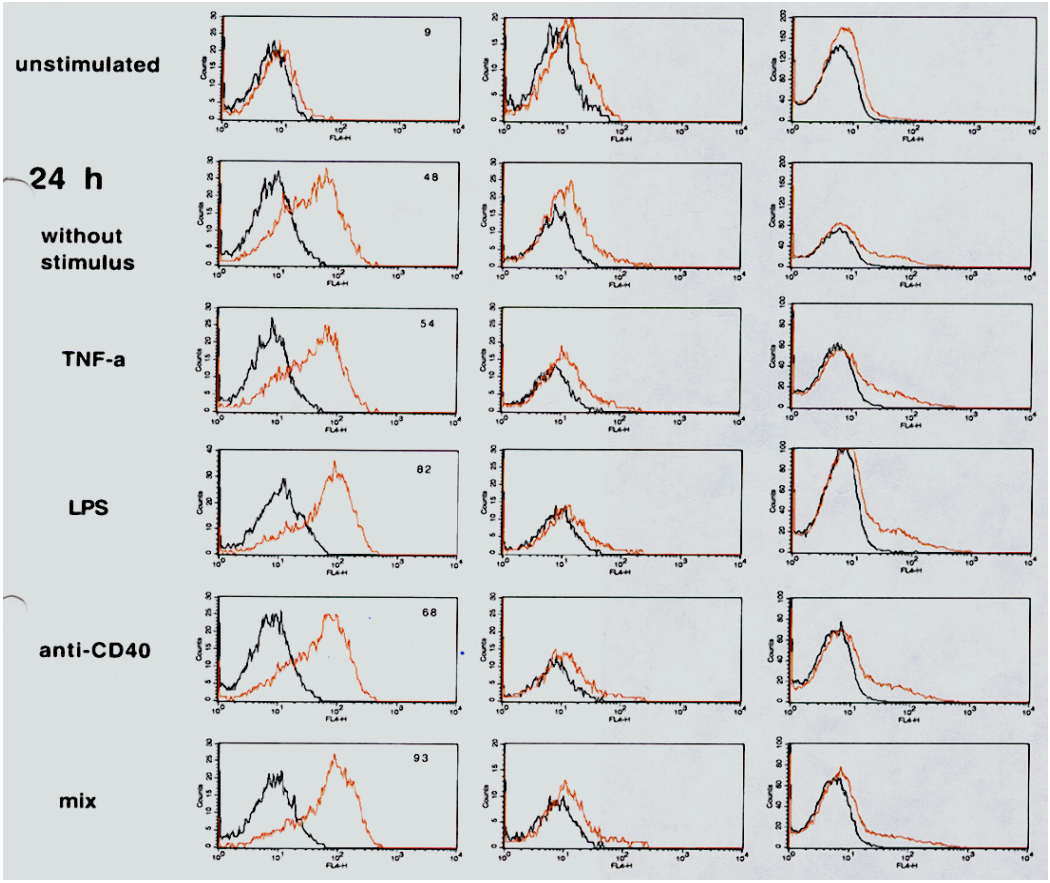


Figure 4.5.1 CD8- DCs express LAG-3 upon activation. Spleen cells were enriched for DCs on Opti-prep® gradients and cells were cultured in the presence or absence of indicated stimuli. (a) CD11c/CD8 staining used to classify DCs (b) LAG-3 staining on DC subpopulations (mAb G15) is shown in red, isotype control in black.

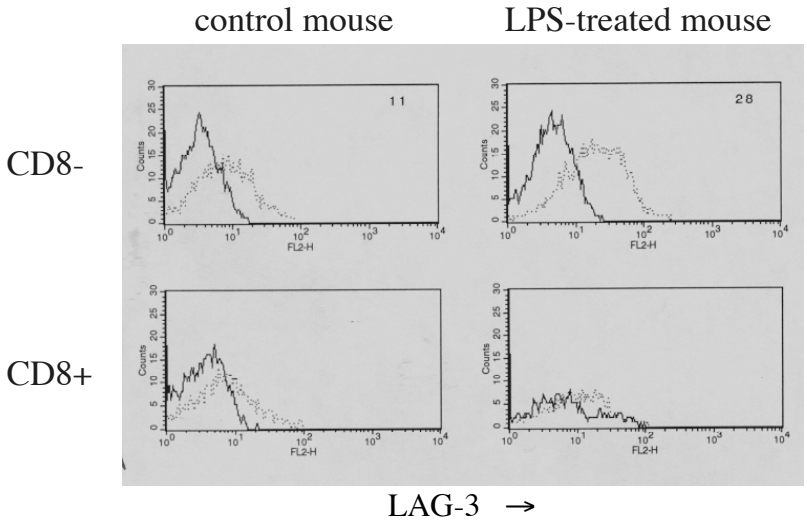


Figure 4.5.2. CD8- DCs up-regulate LAG-3 on their surface upon LPS treatment *in vivo*. Balb/c mice were injected (i.p) with LPS (25 μg/ mouse) and LAG-3 staining was performed on spleen DCs after 24 hours. LAG-3 staining is shown in grey (dotted line), isotype control in black (solid line).

absence of LAG-3 on CD8+ DCs, as well as on thymic T lymphocyte populations (Workman et al., 2002b), one could infer that LAG-3 is not important for developmental events in the thymus; consistent with the observations that positive and negative selection of T cells appeared normal in LAG-3 deficient mice (Miyazaki et al., 1996).

Perhaps the most striking biological difference between CD8+ and CD8- DC populations, however, is the ability of the CD8+ DCs to induce Th1 biased cytokine response in reactive CD4 T cells, whereas CD8- DCs tend to induce a Th2–biased response *in vivo* (reviewed in (Ardavin, 2003; Shortman and Liu, 2002)). Yet, both DC populations are capable of inducing CTL responses and protective antiviral immunity in vivo (Ruedl and Bachmann, 1999). Identification of LAG-3 on CD8-, but not CD8+ DCs, suggests that LAG-3 on DCs might play a role in Th2 biased responses, B-cell activation and plasmablast differentiation.

Subsequently, we also analyzed DC populations present in the LN. After enrichment procedure, dermal, epidermal, direct bone-marrow immigrants, and plasmacytoid DCs (pDCs) were distinguished by staining with CD11c and CD40 Abs ((Ruedl et al., 2000) and C. Ruedl personal communication).

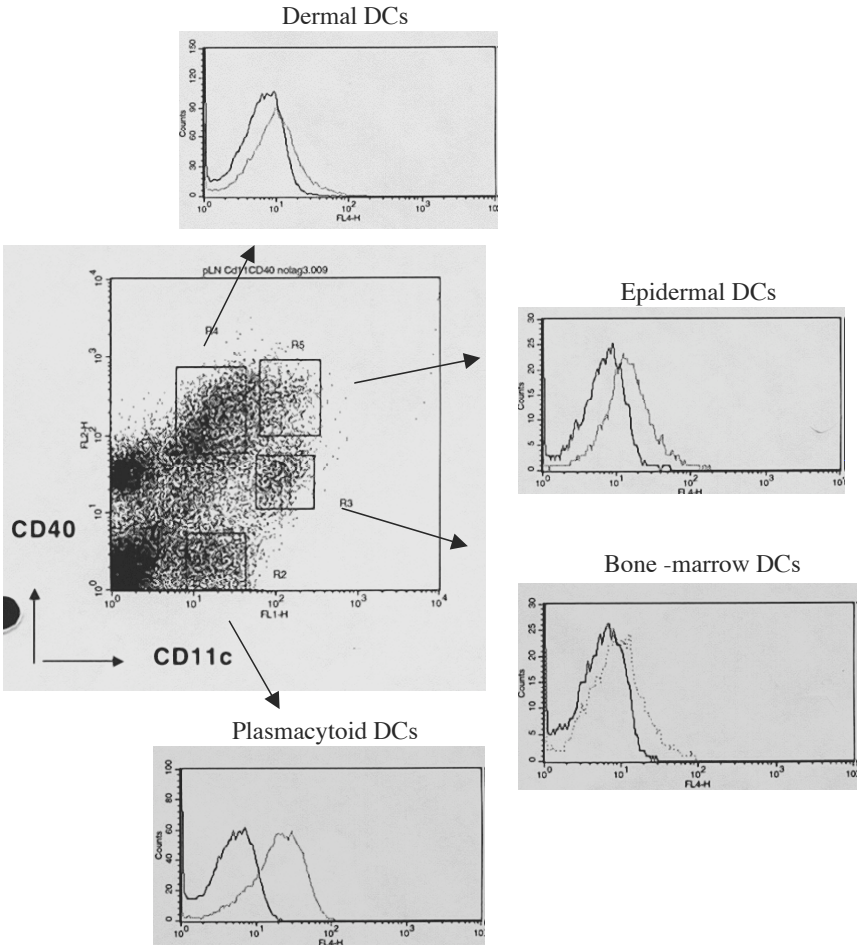


Figure 4.5.3. LAG-3 expression on different subpopulations of LN dendritic cells *ex vivo*. CD11c/CD40/LAG-3 surface staining was performed on peripheral LN population enriched for DC by Opti-prep® gradient. G15 mAb was used for LAG-3 staining. LAG-3 – grey line, isotype control- black line.

As shown in Figure 4.5.3 freshly isolated murine plasmacytoid DCs (CD11c int CD40 neg) stained positively for LAG-3, while dermal (CD11c int CD40 high) epidermal (CD11c high CD40 high) and bone marrow DCs (CD11c high CD40 int) did not. It must be mentioned, however, that intensity of LAG-3 staining of *ex vivo* plasmacytoid cells, varied from experiment to experiment.

Further analysis performed on purified plasmacytoid, dermal and epidermal DCs revealed that pDCs increased their LAG-3 surface levels 100-fold upon stimulation with a Toll

like receptor 9 (TLR 9) ligand CpG (Figure 4.5.4) (Hemmi et al., 2000). Dermal and epidermal DCs were unaffected by this treatment.

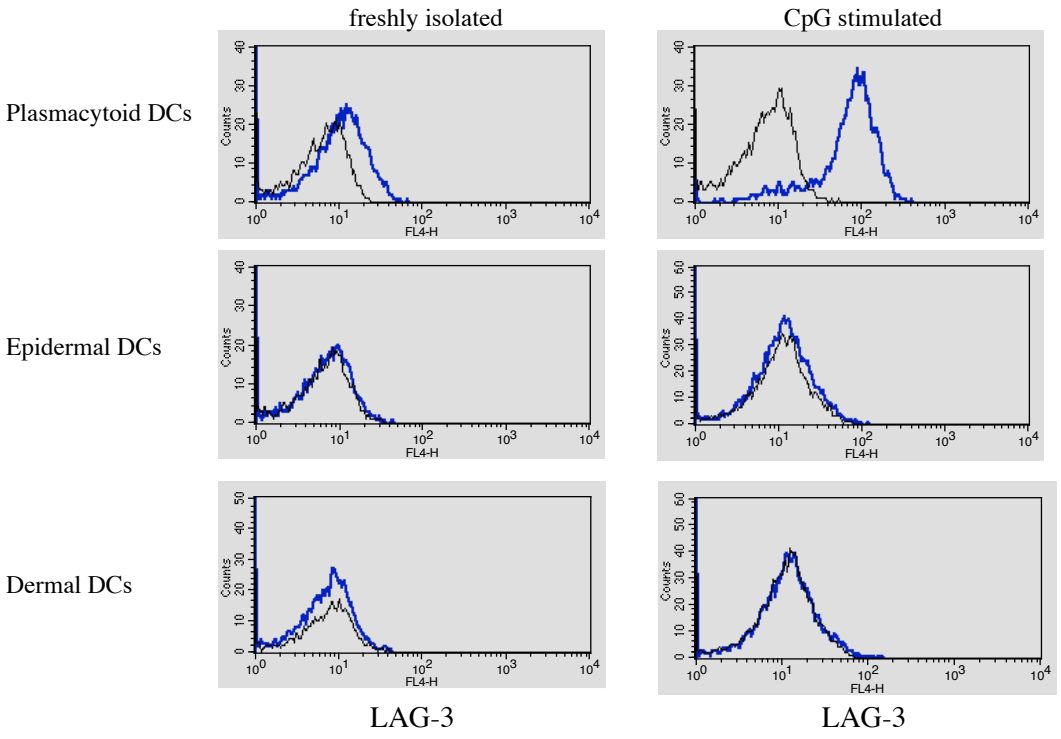
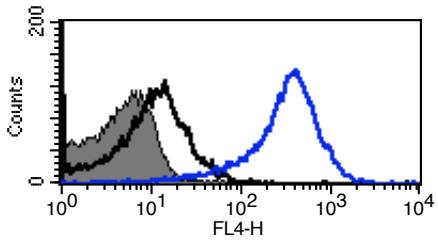


Figure 4.5.4. Plasmacytoid DCs up-regulate LAG-3 expression upon stimulation with CpG. Plasmacytoid, dermal and epidermal DCs were sorted from LN based on CD11c and CD40 staining as shown in Figure 4.5.3. Purified cells were stimulated with CpG 1826 (Ballas et al., 2001) over-night. LAG-3 staining was performed with mAb G15 shown in blue; isotype control shown in black.

The presence of LAG-3 on pDCs suggests that this surface molecule might be involved in anti-viral responses. pDCs, also called interferon-producing cells, circulate as precursor cells in the blood or reside as immature cells in all lymphoid organs. They are the main source of type I interferons in response to viral infection (Asselin-Paturel et al., 2001). Type I interferons in turn, induce resistance to viral replication in all cells. The additional observation, supporting the involvement of LAG-3 in fighting viral infections, is that (as presented in section 4.3) the activated CD8 T cells (the professional killers of infected cells) show the highest levels of LAG-3 among all leukocyte populations examined.

4.6. Indications for a role of LAG-3 in APC function.

Since we were able to detect LAG-3 on the surface of activated APCs (DCs as well as B cells) we wondered whether it is important for APC function. To address this question, we generated LAG-3 positive A20 B cell lymphoma line via retroviral transduction (Fig 4.6.1).



LAG-3

Figure 4.6.1. A20 cells stably transduced with Lag-3 express high levels of surface LAG-3, as detected by surface staining with mAb G15. A20LAG-3 - blue line, A20 cells – black line, negative control- shaded area.

A20 and A20LAG-3 cells were then loaded with increasing concentrations of HA-peptide and used to activate HA-specific CD4 T cell hybridoma – A5 cells (described in more detail in section 4.1). At lower peptide concentrations and for all the time points tested (4 to 14 hours) the higher percentage of A5 cells was activated by A20LAG-3 cells than by A20 cells transduced with an empty vector (Figure 4.6.2), indicating that A20 cells expressing LAG-3 are more efficient in triggering T cell activation. At high peptide concentration and increased stimulation times, however, the effect was abolished, likely due to saturation of the system. These experiments hinted that ectopic expression of high levels of LAG-3 on the surface of B cells can indeed enhance their function as antigen presenting cell.

We then turned to a more physiological situation, that is the *ex vivo* B cells and examined their potential to activate antigen specific T cells. In order to obtain LAG-3 positive and LAG-3 negative B cells, splenic cells from WT or LAG-3 deficient mice were activated on immobilized anti-CD3 and anti-CD28 Abs for 3 days. After this time of culture WT B cells express LAG-3 on their surface (Figure 4.6.3). Live cells were isolated using Ficoll-Paque™ density separation and loaded with various concentrations of OVA peptide.

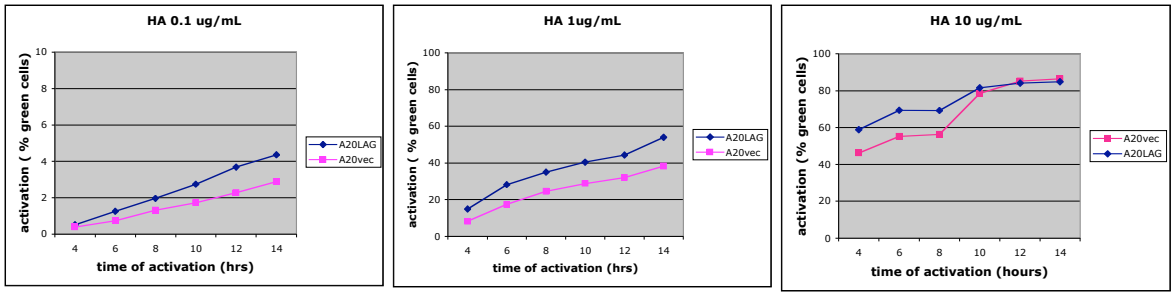


Figure 4.6.2. T cell activation by relevant peptide presented by A20 and A20LAG-3 cells. B cells loaded with HA peptide at indicated concentrations and used to activate A5 cells for varying times. The level of T cell activation was assessed by the percentage of green cells. The degree of T cell activation by A20LAG-3 cells is shown in blue. The level of T cell activation by A20 cells transduced with empty pLXSP vector (A20vec) is shown in pink.

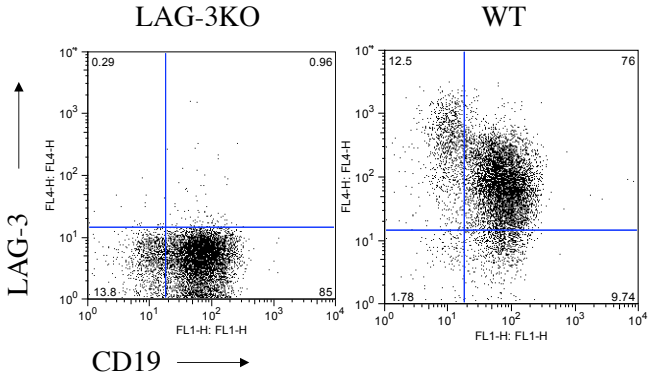


Figure 4.6.3. B cells in co-culture with activated T cells express intermediate levels of LAG-3 on their surface. Day 3 Abs activated splenocyte cultures were stained with anti CD-19 (a B cell marker) and anti-LAG-3 Abs. Both LAG-3 deficient (left panel) and WT (right panel) mouse cells cultures are shown. In both cultures the large majority of cells are B cells; T cell probably die quicker by apoptosis under these efficient stimulatory conditions.

Since the activation state of APCs strongly affects the quality of T-B cell interactions, we also made sure that the levels of co-stimulatory molecules on the surface of LAG-3 positive and LAG-3 negative presenting cells do not differ. As shown in Figure 4.6.4, LAG-3 positive and LAG-3 negative B cells show the same levels of B7.1, B 7.2 and MHC class II. The levels of all three molecules are increased as compared to non-treated splenocytes, making them potent antigen presenting cells.

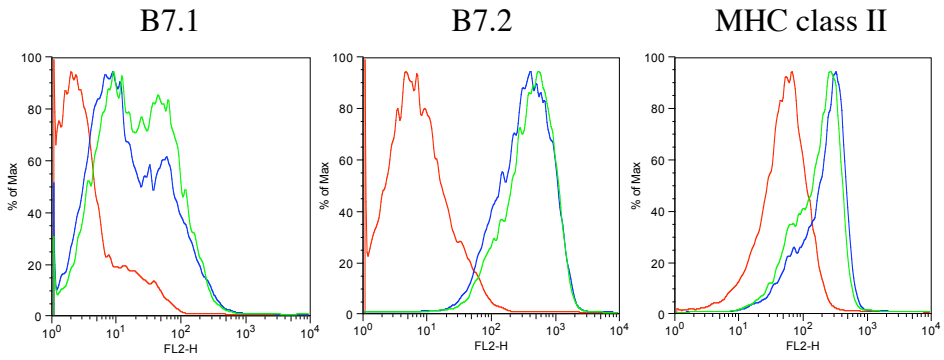


Figure 4.6.4. LAG-3 positive (green line) and LAG-3 negative (blue line) B cells show the same level of activation. Day 3 anti-CD3 and anti-CD28 activated splenocytes (the same culture as in Figure 4.6.4) were stained with anti-B 7.1 (left panel), anti-B7.2 (middle panel) and anti- MHC class II (right panel) Abs. Both LAG-3 positive and LAG-3 negative B cells show increased levels of expression of these activation markers as compared to non-treated splenocytes (red line).

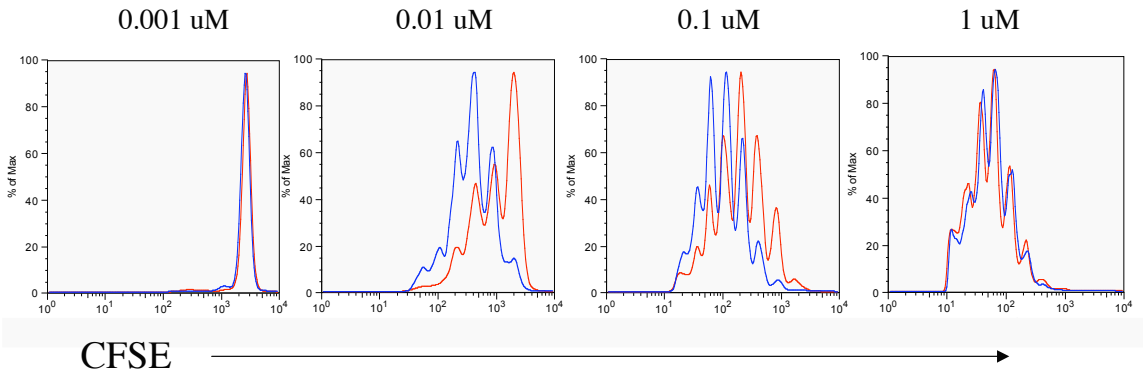
The peptide-loaded B cells were then used to activate CFSE labeled OVA-specific transgenic CD4 T cells, isolated from OT-II mice (Barnden et al., 1998). After 6 days T cell proliferation was determined by CFSE dilution, as shown in Figure 4.6.5a. The strength of the response of transgenic T cells was dependent on the OVA peptide concentrations, over a wide range.

Interestingly, at low peptide concentration (0.01 μM), LAG-3 deficient B cells were less efficient in triggering T cell proliferation; only 50% of T cells activated by LAG-3 deficient B cells divided, while more than 94% T cells divided when they were activated with LAG-3 positive B cells. At higher peptide concentration (0.1uM), the proliferation of T cells activated by LAG-3 deficient B cells was also clearly delayed, but much lower percentage of cells remained undivided (Figure 4.6.5 b).

When presenting cells were flooded with the OVA peptide (1uM), the difference in antigen presentation by LAG-3 positive and LAG-3 negative B cells was not observed. This peptide concentration, however, led to the over-stimulation of T cells and increased T cell death, unlikely resembling a healthy *in vivo* activation process.

The observations that B cells expressing LAG-3 were more efficient in stimulating antigen specific T cell proliferation than cells lacking LAG-3 shows that LAG-3 surface expression can affect T cell triggering in trans.

a)



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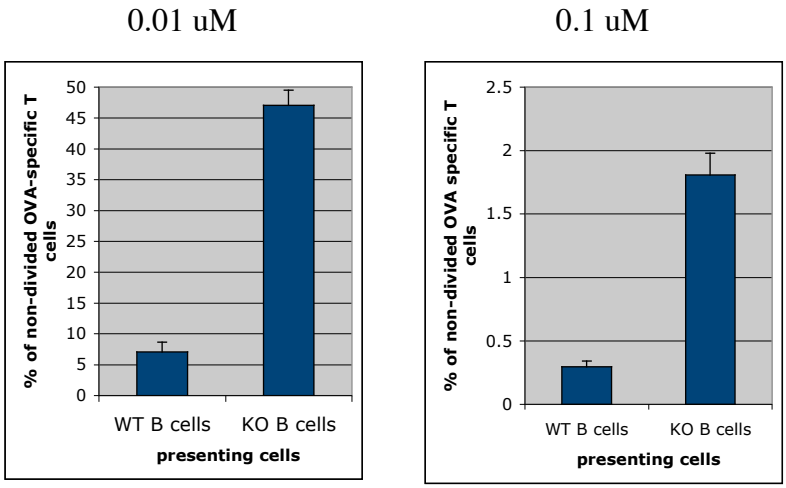

Figure 4.6.5. Surface expression of LAG-3 by B cells affects their ability to activate T cells. (a) OVA–specific, CFSE labelled CD4 T cells were activated with LAG-3 positive (in blue) or LAG-3 negative (in red) B cells loaded with various concentration of OVA peptide. Peptide concentration used to activate B cells is indicated in μM. (b) Stimulation of T cells with LAG-3 KO B cells loaded with 0.01 uM or 0.1 uM OVA peptide results in higher percentage of non-proliferating T cells as compared to WT B cells, the error bars represent standard deviation among triplicates.

Whether LAG-3 enhances Ag presentation, acts as a novel co-stimulatory molecule or stabilizes the immunological synapse remains to be clarified. As LAG-3 is known to form homodimers, the interaction of LAG-3 on APCs with LAG-3 on T cells can be considered as a possible mode of action.

Nevertheless, the above data indicates the LAG-3 involvement in APC function.

5. GENERAL DISCUSSION AND FUTURE PROSPECTS

At the beginning of this study LAG-3 was considered to be a T and NK cell specific molecule, involved in negative regulation of T cell responses. The work presented here shows that LAG-3 expression in the immune system of a mouse is much broader. B cells and DCs (especially pDCs) can be induced to express LAG-3 and LAG-3 positive B cells are better APCs. These findings indicate a more complex role for LAG-3 in the immune system possibly also as a novel co-stimulatory molecule. One of the practical benefits of this work could be a new immunization protocol involving LAG-3 transfected APCs. Consistent with this idea, soluble LAG-3 has been previously shown to be a potent immunostimulant for inducing antigen- or tumor-specific CTL and CD4 Th1 responses (El Mir and Triebel, 2000; Prigent et al., 1999).

LAG-3 expression on B cells is mediated by a soluble factor. Unfortunately, we have failed to identify this factor among the known cytokines we tested. More intensive studies are required to solve this issue. Potentially, it is a novel lymphokine involved in T-B cell communication. Identification of a T cell line producing this factor would greatly facilitate subsequent analysis.

It is clear that MHC class II is a ligand for LAG-3. However, since activated CD4 and CD8 T cells, NK cells and APCs are all positive for LAG-3 it can be hypothesized that LAG-3 has alternative ligand(s). To research this area one could produce LAG-3 immunoglobulin (Ig) fusion proteins and use these as specific reagents in flow cytometry and in immunohistology to detect any binding to cells or tissues derived from MHC class II deficient mice. Further analysis could then lead to the characterization of the new putative ligand(s).

The potential multiple roles of LAG-3 in the immune system are further emphasised by the fact that LAG-3 exists not only in membrane bound form but also in a soluble form. Initially, soluble human LAG-3 was identified in the serum of healthy individuals (Annunziato et al., 1996). We show that soluble LAG-3, with the approximate molecular weight of 53kDa, can be easily detected in the supernatant of murine splenocytes

activated by TCR ligation (results section 4.4 Figure 4). Soluble LAG-3 can be produced by activated CD4 as well as activated CD8 T cells. It was found in high concentrations in culture supernatants, reaching 2 ug/ml, as determined by ELISA (data not shown). Concominantly, Li and co-workers showed that soluble LAG-3 (with the predicted molecular weight of 54 kDa) is present in the supernatants of splenocyte cultures from OT-II transgenic mice activated with OVA (Li et al., 2004). Moreover, they detected low amounts of soluble LAG-3 in the sera of WT mice (~180 ng/ml). Surprisingly, this amount of serum LAG-3 was also detectable in RAG-1- deficient mice (mice lacking T cells and B cells), suggesting that NK cells might be the main source of soluble LAG-3 in these mice. Alternatively, soluble LAG-3 could be produced by DCs (in particular pDCs).

Furthermore, in the light of the classical regulatory T cell assay results presented here, LAG-3 involvement in regulatory T cell function becomes questionable. LAG-3 should not be used as a specific marker for T cell activation, nor can it provide the means for distinguishing the regulatory T cell population.

Our results suggest that, indeed T cell function can also be controlled extrinsically by LAG-3, but when it is expressed on APCs, rather than on T regs.

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PUBLICATIONS

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