The CD4-like molecule LAG-3, biology and therapeutic applications

Sophie Sierro, Pedro Romero & Daniel E Speiser

Lausanne University Hospital, Avenue Pierre-Decker 4, 1011 Lausanne, Switzerland

Importance of the field: Promising immunotherapeutic agents targeting co-stimulatory pathways are currently being tested in clinical trials. One player in this array of regulatory pathways is the LAG-3/MHC class II axis. The lymphocyte activation gene-3 (LAG-3) is a negative co-stimulatory receptor that modulates T cell homeostasis, proliferation and activation. A recombinant soluble dimeric form of LAG-3 (sLAG-3-Ig, IMP321) shows adjuvant properties and enhances immunogenicity of tumor vaccines. Recent clinical trials produced encouraging results, especially when the human dimeric soluble form of LAG-3 (hLAG-3-Ig) was used in combination with chemotherapy.

Areas covered in this review: The biological relevance of LAG-3 in vivo. Preclinical data demonstrating adjuvant properties, as well as the improvement of tumor immunity by sLAG-3-Ig. Recent advances in the clinical development of the therapeutic reagent IMP321, hLAG-3-Ig, for cancer treatment.

What the reader will gain: This review summarizes preclinical and clinical data on the biological functions of LAG-3.

Take home message: The LAG-3 inhibitory pathway is attracting attention, in the light of recent studies demonstrating its role in T cell unresponsiveness, and Treg function after chronic antigen stimulation. As a soluble recombinant dimer, the sLAG-3-Ig protein acts as an adjuvant for therapeutic induction of T cell responses, and may be beneficial to cancer patients when used in combination therapies.

Keywords: adjuvant, cancer, immune response, LAG-3, T cell

1. Introduction

The immune system has evolved many mechanisms to fine-tune an immune response, allowing for efficient rejection of invading pathogens with as little damage as possible of host tissues. This amazing equilibrium is possible through multiple regulatory pathways working singly or together. One molecule taking part in this well orchestrated dance is the lymphocyte activation gene-3, more commonly called LAG-3.

2. Biology of LAG-3

2.1 Molecular properties

A member of the immunoglobulin superfamily, the LAG-3 (CD223) is a CD4-like protein, which like CD4, binds to MHC class II molecules. The gene coding for the LAG-3 protein lies adjacent to the gene coding for CD4 on human chromosome 12 and shares approximately 20% identity with the cd4 gene. Comparisons of their gene organization [1] and protein domain structure suggest that LAG-3 and CD4 are
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2.2 Biological functions

2.2.1 T cell inhibition

The inhibitory role of LAG-3 was first shown in vitro. Improved T cell proliferation in the presence of LAG-3-blocking mAbs established its role as negative regulator involved in the decline of T cell responses. Further studies demonstrated that simultaneous cross-linking of the T cell receptor (TCR) and LAG-3 on activated T cells resulted in lower calcium release when compared with TCR cross-linking alone [19]. In addition, LAG-3 overexpression or inhibition of LAG-3 surface cleavage lead to impaired antigen-driven T cell proliferation without however inducing T cell death [6].

LAG-3 knockout mice generated in the late 1990 [20] that displayed an apparently normal phenotype highlighted the fact that the effects of LAG-3 are rather subtle and that the molecule is likely to be involved in the fine tuning of the immune response. In-depth analysis showed that LAG-3 plays a role in T cell homeostasis as well as in T cell proliferation and T cell effector functions [21,22].

2.2.2 Role in CD4+ T cells

The physiological ligand for LAG-3 is MHC class II to which it binds with a higher affinity than CD4 [23]. Cross-linking of LAG-3 on T cells impairs TCR-mediated activation in CD4+ T cells resulting in decreased calcium flux [19], lower IL-2 production and reduced production of Th1 type cytokines [24]. Additionally, ectopic expression of the full-length LAG-3 molecule in naïve CD4+ T cells confers regulatory activity to the cells, while expression closely related and derived from a common ancestor [2]. Both CD4 and LAG-3 have four extracellular Ig-like domains with conserved structural motifs throughout (D1 to D4 domains) [2].

Although these two molecules are closely related, LAG-3 binds to MHC class II with a higher affinity than CD4 and at a distinct site from CD4 [3]. The two molecules are also differentially localized and exert very distinct functions. Whereas CD4 is mainly expressed at the cell surface with only a small fraction residing intra-cellularly, roughly half of the LAG-3 molecules are retained inside the cell close to the microtubule-organizing centre (MTOC) [4]. Co-localization of LAG-3 with Rab11b suggests a possible rapid translocation of the molecule to the plasma membrane. Unlike CD4, LAG-3 is mainly expressed after lymphocyte activation. Functionally, CD4 acts as a positive co-stimulatory molecule whereas LAG-3 falls in the negative co-stimulatory group (inhibitory co-receptors) of molecules (such as, for example, B and T lymphocyte associated (BTLA), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1)) [5].

At the cell surface, LAG-3 is expressed as a dimer [3], and LAG-3 cell surface expression is regulated by extracellular cleavage mediated via two disintegrins/metalloproteases ADAM10 and ADAM17 [6]. LAG-3 expression has been detected in many different cells of the hematopoietic lineage, such as plasmacytoid dendritic cells (pDCs) [7], B [8], NK [9], NKT [10], γδ T cells [11], activated and regulatory T cells [12] and tumor infiltrating lymphocytes [13,14].

The function of LAG-3 is dependent on its binding to MHC class II and signaling through its cytoplasmic domain. LAG-3 has a unique cytoplasmic tail, which differs greatly from CD4, containing two particular motifs. The first, a unique KIEELE motif (conserved between human and mice) is essential for its inhibitory cell intrinsic function [15]. The second motif is an unusual ELP (glutamic acid/proline) repeat that associates with LAG-3-associated protein (LAP) [16] and may play a role in LAG-3 localization.

A monomeric soluble form of LAG-3 is found in the serum of patients with inflammatory disorders. Increased levels of soluble LAG-3 were found in patients that underwent successful tuberculosis treatment as well as in patients protected from disease [17]. Similarly, in a subset of breast cancer patients, a detectable level of LAG-3 in the serum at diagnosis was associated with a longer disease-free survival as well as overall disease-specific survival [18]. In patient serum, the soluble form of LAG-3 is derived from an alternatively spliced molecule composed of the D1–D3 domains. In mice, experimental evidences suggest that the soluble form of LAG-3 obtained by cleavage of cell surface LAG-3 by metalloproteases does not play a regulatory role and is likely to be a waste product [6]. In vivo, it is thought that membrane tethering is required to maintain a high-affinity form. This system provides a unique mechanism for retaining the high-affinity MHC class II interaction required for LAG-3 function while safely rendering soluble LAG-3 in sera innocuous [6].

This box summarizes key points contained in the article.

- LAG-3, a CD4-like molecule is expressed by T cells, B cells, NK cells and plasmacytoid dendritic cells (pDCs).
- LAG-3 is upregulated following T cell activation, and modulates T cell function as well as T cell homeostasis.
- Subsets of conventional T cells that are anergic or display impaired functions express LAG-3. LAG-3+ T cells are enriched at tumor sites and during chronic viral infections.
- Recombinant soluble LAG-3 expressed as a dimer (IMP321) shows adjuvant properties.
- Current clinical trials in cancer patients show encouraging results of treatment with IMP321, and warrant further efforts to elucidate the biology of LAG-3, as well as the therapeutic mechanisms of IMP321.
of a cytoplasmic truncated LAG-3 mutant does not [15,21]. In the presence of LAG-3, CD4+ T cell proliferation is reduced (average cell division lower as compared with LAG-3+/+ cells) particularly at low antigen concentration. Reduced proliferation is accompanied by diminished IL-2 and IFN-γ production [6].

In humans, effector CD4+ T cells express MHC class II upon T cell activation. MHC class II cross-linking during activation of CD4+ T cell clones using a soluble dimeric form of LAG-3 inhibited T cell proliferation and cytokine secretion. In contrast, LAG-3 blockade using an antagonist LAG-3 antibody during antigen-specific T cell stimulation of primary human CD4+ and CD8+ T cells led to increased T cell proliferation and augmented function [25].

These studies support the notion of a T cell autonomous inhibitory signaling pathway. Thus it has been proposed that the interaction between MHC-class-II-bearing antigen presenting cells (APCs) and LAG-3+/+ T cells induces a T cell intrinsic inhibitory signaling pathway, accounting for the decreased T cell activation observed. Furthermore the tight regulation of LAG-3 expression at the cell membrane via storage in intracellular vesicles and surface cleavage by metalloproteases probably limits autonomous inhibitory T cell signaling [6].

### 2.2.3 Role of LAG-3 in regulatory T cells in mouse models

T regulatory (Treg) cells can be broadly divided in two groups: naturally occurring Treg and adaptive or induced Treg. Natural Treg are generated in the thymus, whereas induced Treg develop in the periphery from naïve CD4+ or CD8+ T cells following antigen-specific activation in an immunosuppressive environment. Microarray analysis revealed that LAG-3 mRNA is expressed in large amounts by naturally occurring CD4+CD25+ Treg despite the fact that little LAG-3 protein can be detected at the cell surface by flow cytometry analysis. Yet, following stimulation of natural Treg cells with anti-CD3 in the presence of APCs, LAG-3 protein levels are strongly upregulated and can be detected at the cell surface [12].

In a mouse model of induced Treg, where HA transgenic CD4+ specific T cells are rendered anergic following their transfer into transgenic mice expressing HA in multiple epithelial tissues, elevated level of LAG-3 protein was found to be expressed by induced HA-CD4+ Treg [12]. These induced Treg, not derived from naturally occurring CD4+CD25+ Treg, showed maximal in vitro suppressive functions when expressing LAG-3. In vivo, administration of a LAG-3-blocking antibody abolished their suppressive capacity. Additional experiments compared the suppressive capacities of LAG-3-/- induced Treg and LAG-3+/+ induced Treg. Both sets of cells could inhibit peptide-induced proliferation of antigen-specific CD4+ T cells. However when high concentrations of peptide were used to induce antigen-specific T cell proliferation, three times more LAG-3-/- Treg were required to suppress to the same extent as LAG-3+/+ Treg. This model suggests that LAG-3 expression is required to provide maximal Treg activity. A correlation between LAG-3 mRNA level and IL-10 mRNA level was also found. Altogether, LAG-3 plays a role in the suppression mediated by induced Treg, with LAG-3 expression required for maximal regulatory capacity.

In naïve animals, LAG-3 expression on CD4+ T cells is mainly restricted to the CD25+CD45RBhigh subset thought to belong to the group of induced Treg cells. These CD4+CD25-CD45RBhigh LAG-3 forkhead box P3 (FoxP3)+ T cells are found in small numbers in the spleen, mesenteric lymph nodes (MLN), and inguinal lymph nodes and in higher numbers in Peyer’s patches (PP). Following anti-CD3/anti-CD28 stimulation in vitro, this cell subset proliferated poorly, produced elevated amounts of IL-10 and IFNγ, did not express the Treg marker FoxP3, and possessed only weak suppressive activity. However, in vivo, in a model of T-cell-mediated colitis driven by enteric bacteria in lymphopenic animals following the transfer of naïve CD4+ CD45RBhigh cells, CD4+ CD25+ LAG-3+ could inhibit the development of colitis in an IL-10-dependent manner [26]. In contrast to CD4+CD25+ natural Treg, the development of CD4+ CD25+ LAG-3+ T cells does not appear to require high-affinity interaction with selecting peptide MHC-ligands expressed in the thymus, but rather they develop following exposure to environmental microflora and APCs or exposure to self antigen [12,26].

Altogether these results suggest that LAG-3 plays a role in the suppression mediated by natural, as well as induced, Treg. Furthermore, the sole expression of LAG-3 on naïve CD4+ T cells can confer regulatory functions without inducing the expression of genes associated with regulatory T cells such as FoxP3, CTLA-4, glucocorticoid-induced TNFR-related protein (GITR) or CD25, suggesting that LAG-3 may mediate suppression independently of the FoxP3 pathway.

### 2.2.4 LAG-3 in human Tregs

In humans the expression of LAG-3 identifies a population of CD4+ CD25+ FoxP3+ Treg in the peripheral blood. This subpopulation is expanded in the blood and at the tumor site in patients with advanced cancers [27]. In vitro, co-culture of Treg and dendritic cells (DCs) yielded DCs with a semi-mature phenotype. Blocking LAG-3 during the co-culture, inhibited CCR7, CXCR4 and HLA-DR upregulation and led to reduced expression of CD80, CD86, IL-6, TNF-α and IL-8 by DCs [28]. The change in phenotype observed when blocking LAG-3 suggest that LAG-3 induced a partial maturation of DCs. However, maturation-associated changes triggered by LAG-3 may be modulated by the engagement of other inhibitory molecules expressed by Treg. The integration of different signals delivered to DCs by Treg is likely to differ from receiving a single LAG-3 signal. In vivo, LAG-3 may contribute to Treg function through control of the steady-state DC migration, as Treg-conditioned DCs have been shown to trigger abortive, tolerance-inducing responses upon antigen presentation to T cells (Figure 1).
2.2.5 Role of LAG-3 in CD8+ T cells

As for CD4+ T cells, naïve CD8+ T cells express low levels of LAG-3, however, this expression dramatically increases following antigen stimulation [11]. Recent studies have highlighted the fact that exhausted CD8+ T cells following chronic viral infections express multiple inhibitory receptors (such as PD-1, BTLA, or CD160) in conjunction with other inhibitory co-receptors such as CTLA-4, GITR, PD-1, or CD160 may enhance T cell intrinsic suppressive activity. MHC class II cross-linking, in addition to other inhibitory signals provided through the simultaneous engagement of PD-L1, HVEM or GITRL, may induce a tolerogenic state of APCs. Activated (CD4+ or CD8+) T cells, which upregulate LAG-3, receive cell-intrinsic signals via LAG-3 cross-linking, leading to reduced proliferation. MHC class II cross-linking by LAG-3 may induce a negative signal on APCs, possibly over-ridden by activatory co-receptors (e.g., CD40 and CD28) expressed on activated CD4+ T cells. Positive signals through CD40L and CD80/CD86, in conjunction with MHC class II cross-linking may lead to APC activation. APC: Antigen presenting cell; DC: Dendritic cell; TCR: T cell receptor.

Figure 1. Membrane bound LAG-3 as inhibitory co-receptor: Antigen-specific T cells are activated by antigen presenting cells (APC) presenting cognate peptide. A. Tregs (CD4+ or CD8+) expressing LAG-3 cross-link MHC class II molecules on APCs. LAG-3 engagement in conjunction with other inhibitory co-receptors such as CTLA-4, GITR, PD-1, BTLA, or CD160 may enhance T cell intrinsic suppressive activity. MHC class II cross-linking, in addition to other inhibitory signals provided through the simultaneous engagement of PD-L1, HVEM or GITRL, may induce a tolerogenic state of APCs. B. Activated (CD4+ or CD8+) T cells, which upregulate LAG-3, receive cell-intrinsic signals via LAG-3 cross-linking, leading to reduced proliferation. MHC class II cross-linking by LAG-3 may induce a negative signal on APCs, possibly over-ridden by activatory co-receptors (e.g., CD40 and CD28) expressed on activated CD4+ T cells. Positive signals through CD40L and CD80/CD86, in conjunction with MHC class II cross-linking may lead to APC activation.

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Recent studies have highlighted the fact that exhausted CD8+ T cells following chronic viral infections express multiple inhibitory receptors (such as PD-1, CD160 and CD28). Blocking the PD-1/programmed cell death ligand 1 (PD-L1) inhibitory pathway in vivo can lead to a dramatic reduction of viral load in chronically infected mice [29]. Among these inhibitory co-receptors, LAG-3 was shown to be expressed at high levels after lymphocytic choriomeningitis virus (LCMV) clone 13 infection [30]. Surprisingly, however, blocking LAG-3 in vivo during chronic LCMV infection had no or only a marginal effect on virus load [30,31]. Similarly, infection of LAG-3−/− knockout or wild-type mice with LCMV showed similar viral titers in all of the organs tested [31]. Furthermore, LAG-3−/− mice mounted normal virus-specific CD4+ and CD8+ T cell responses, suggesting a non-essential role for LAG-3 in this particular setting [31]. Yet interestingly, blockade of the PD-1/PD-L1 pathway combined with LAG-3 blockade improved viral control when compared with PD-L1 blockade alone [30].

The role of LAG-3 was also studied in a self-tolerance/tumor model where transgenic CD8+ T cells were rendered unresponsive/anergic in vivo by encounter with their cognate self-antigen. Interestingly, these anergized CD8+ T cells also expressed high levels of LAG-3. LAG-3 blockade or LAG-3 deficiency on the transgenic specific CD8+ T cells enhanced T cell proliferation, T cell recruitment and effector functions at the tumor site [13]. These results therefore suggest that LAG-3 may be involved in the maintenance of a CD8+...
T cell tolerogenic state. Surprisingly in this model, CD8+ T cells that co-expressed LAG-3 and low levels of PD-1 produced more cytokines and killed better than LAG-3−PD-1low cells [32]. These apparently contradictory results will need to be addressed in future studies.

When re-stimulating PBMC from BCG-vaccinated patients in vitro with Bacillus Calmette-Guérin (BCG), Joosten et al. described the occurrence of a CD8+ Treg subset expressing LAG-3. These CD8+LAG-3+ cells could suppress both antigen and mitogen induced T cell responses, partly through the secretion of chemokine (C-C motif) ligand 4 (CCL4) [33]. More recently another subset of CD8+LAG-3+ T cells specific for Mycobacterium tuberculosis and human leukocyte antigen (HLA)-E was identified. These cells possessed cytotoxic as well as regulatory properties and suppressed via membrane bound TGF-β [34].

In human ovarian cancer, tumor-infiltrating New York esophageal squamous cell carcinoma 1 (NY-ESO-1)-specific CD8+ T cells expressed high levels of PD-1 with a proportion of these cells also co-expressing LAG-3 [34]. Co-expression of PD-1 and LAG-3 identified a sub-population of T cells with diminished capacity to produce IFN-γ and TNF-α. In vitro, blockade of LAG-3 alone or in combination with PD-1 did not enhance cytokine production by tumor-infiltrating lymphocytes (TILs) in short-term assays. Interestingly, blocking LAG-3 or PD-1 for prolonged periods (10−14 days) enhanced the number of NY-ESO-1 CD8+ T cells but not their function whereas combined blockade of LAG-3 and PD-1 increased both the T cell frequency and IFN-γ-producing cells. LAG-3 expression by CD8+ T cells could be induced following re-stimulation of PBMC with tumor-derived APCs. Moreover LAG-3 expression could be induced in an antigen-independent manner in presence of IL-10 and IL-6 [14].

Collectively these data support the view that similar to the situation in CD4+ T cells, LAG-3 expressed by CD8+ T cells is a negative regulator. Evidence collected from mouse models suggests that LAG-3 is induced following prolonged antigen, particularly self-antigen, stimulation in a non-immunogenic environment. The presence of this negative regulator influences T cell proliferation and T cell function. In vitro studies carried out using human samples also show similar effects, with enhanced regulatory activities and lower proliferative potential of LAG-3-expressing CD8+ T cells.

2.2.6 Role of LAG-3 in pDC homeostasis

Recently, it was reported that in mice, plasmacytoid DCs constitutively express LAG-3. Surprisingly, pDCs express far higher levels of LAG-3 than Treg and activated T cells. This expression was revealed by direct ex vivo FACs staining. In the same way that LAG-3 plays a role in T cells homeostasis, LAG-3 expression also influences pDCs homeostasis. Additionally LAG-3 expression by pDCs modified their expansion following stimulation. LAG-3− pDCs showed enhanced in vitro expansion following CpG stimulation but did not differ in the expression of activation markers when compared with wild-type pDCs [7]. In addition, T cells undergo greater homeostatic proliferation in the presence of LAG-3− pDCs suggesting a homeostatic reciprocity between T cells and pDCs where each population may influence the homeostasis of the other. However in humans LAG-3 does not seem to be expressed on peripheral blood pDCs (F Triebel, pers commun).

2.2.7 Inhibition of APC through LAG-3

One aspect of the LAG-3−MHC class II interaction that has also been investigated is the reverse (retrograde) inhibitory/activatory signaling through MHC class II on APCs. Cross-linking of MHC class II molecules at the surface of DCs inhibits DC maturation [35,36]. So not surprisingly, in mice engagement of MHC class II molecules on DCs by antigen-specific Treg expressing LAG-3, in the presence of their cognate peptide, inhibited CD86 upregulation and diminished IL-12 secretion [35]. In this study, Treg inhibited DC maturation in an antigen-dependent manner only when expressing LAG-3. Expression of LAG-3 lacking its cytoplasmic tail was sufficient to confer regulatory activity to T cells, consistent with the notion that reverse signaling through MHC class II in DCs rather than LAG-3 signaling in T cells is responsible for inhibition of DC maturation. These data suggest that the LAG-3−MHC class II interaction can function as a bi-directional inhibitory pathway (Figure 1).

3. Therapeutic applications of soluble recombinant dimeric LAG-3 protein

Blocking LAG-3 using monoclonal antibodies as well as harnessing the potential of LAG-3 to activate DCs are under development as therapeutic applications. Interfering with the LAG-3/MHC class II pathway may help to prime or potentiate pre-existing T cell responses to viral or tumor antigens and also provide adjuvant activity in the case of therapeutic vaccination (Figure 2). A soluble recombinant dimeric LAG-3 protein (LAG-3−Ig) has been tested in pre-clinical models. The results provide the rational for use of this molecule in the clinics, particularly in cancer patients.

3.1 Adjuvant activity of soluble recombinant dimeric LAG-3 protein

In humans, when used as a soluble recombinant Fc fusion protein, the human dimeric soluble form of LAG-3 (hLAG-3−Ig) induces DC activation and provides immune adjuvant activity. This is in clear contrast to the inhibitory activity of the membrane-bound form of LAG-3.

MHC class II engagement on immature monocyte derived DCs (MoDCs) by hLAG-3−Ig induces DC maturation, whereas MHC class II cross-linking using anti-MHC class II-specific antibodies does not [36]. The reasons for this difference are still unclear. However, it could be shown that MHC class II engagement by hLAG-3−Ig or anti-MHC class II Abs on MoDCs triggered distinct downstream signaling events [37].
Healthy donors and cancer patients were analyzed to assess the effects of hLAG-3-Ig. The addition of hLAG-3-Ig in \textit{in vitro} T cell cultures enhanced the proliferation of antigen specific T cells such as Influenza, Melan-A/melanoma antigen recognized by T-cells 1 (MART-1) or survivin-specific CD8\(^+\) T cells [38]. It also induced upregulation of activatory molecules such as CD83, CD86 and CD40, and secretion of pro-inflammatory cytokines and chemokines (TNF-\(\alpha\), CXCL8, CXCL10, CCL2, CCL9) by APCs. Activated APCs can then boost preexisting immune responses. In combination with antigens or chemotherapy, sLAG-3-Ig also increases phagocytosis and antigen presentation. In conjunction with chemotherapy, additional factors may contribute to APC activation, enhancing the therapeutic effect of sLAG-3-Ig.

Further analysis demonstrated that LAG-3 expressed by activated specific CD4\(^+\) T cells delivers a co-stimulatory signal which contributes to MoDCs activation [39]. LAG-3 and CD40 L expressed by activated T cells co-operate to improved MoDC maturation. This cooperation stimulated IL-12p70 production and enhanced TNF-\(\alpha\) and IL-6 secretion by MoDCs when compared with hLAG-3-Ig stimulation alone. Although incubation with hLAG-3-Ig induced some degree of activation, MoDCs conserved their ability to phagocytose...
apoptotic melanoma cells, suggesting that only a partial maturation state is induced. In vitro, DCs exposed to hLAG-3–Ig secreted CXCL10, CXCL9, CCL2 and CCL8 and migrated and in response to CCL19 but not in response to CCL5. At suboptimal doses of CD40L, hLAG-3–Ig acted as a strong co-stimulatory factor and induced full DC activation. Blocking the LAG-3/MHC class II interaction between activated CD4⁺ T cells and DCs decreased cytokine release by DCs. This help may be particularly important in the absence of sustained TLR-mediated signals and when CD40–CD40L interaction is limited, such as in the case of tumors [40].

Interestingly, hLAG-3–Ig binds less than 10% of MHC class II⁺ cells in PBMC. Among these cells, two different cell subsets were identified: the first displays a myeloid DC phenotype and the second subset belongs to the monocyte lineage. Following attachment of the hLAG-3–Ig protein, these cells rapidly produced TNF-α and CCL4. In turn, this induced IFN-γ and TNF-α production by NK cells and a subset of terminally differentiated effector CD8⁺ T cells [41].

3.2 Anti-tumor responses in pre-clinical models
Vaccination studies carried out in mice with particulate hepatitis B surface antigen (HBsAg) or soluble Ova protein in the presence of the mouse soluble recombinant mLAG-3–Ig protein induced antigen-specific CD8⁺ and CD4⁺ T cell responses [42].

Injection of live tumor cells in combination with murine LAG-3–Ig (mLAG-3–Ig) slowed the rate of tumor growth in vivo. Likewise, vaccination of mice bearing small established tumors with irradiated tumor cells combined with mLAG-3–Ig also was immunogenic and delayed tumor progression [43].

DNA vaccination together with mLAG-3–Ig appeared to synergize in a transgenic tumor model of HER-2/neu mammary gland carcinoma. This combination therapy inhibited the growth of mammary gland tumors when treatment was initiated early, before the appearance of tumors. More interestingly, mice vaccinated when lesions were already detectable, showed delayed tumor progression as well as decreased tumor multiplicity [44].

Combining GM-CSF-secreting tumor cell immunotherapy with mLAG-3–Ig improved tumor specific CD4⁺ and CD8⁺ T cells responses and prolonged the survival of B16 tumor-bearing mice in a therapeutic setting [45]. The addition of mLAG-3–Ig to the therapy accelerated the T cell response and increased the number of lymphocytes infiltrating the tumor as well as their function.

Altogether these studies indicate that combining the soluble recombinant LAG-3 protein with various immunization protocols enhanced anti-tumor immune responses and improved tumor control. This adjuvant effect used in a tumor setting seems to enhance especially CD8⁺ T cells responses.

3.3 Preclinical testing of a clinical-grade dimeric soluble LAG-3 (sLAG-3)
A good manufacturing practice (GMP)-grade of the hLAG-3–Ig protein (also called IMP321) was produced for preclinical and clinical development. Safety evaluation and toxicology tests showed that IMP321 is well tolerated even at high doses. Functional tests demonstrated that the biological effects of the GMP-manufactured IMP321 were preserved [46]. Experiments assessing biological activity revealed that IMP321 enhanced cross-presentation of antigens, as well as DC maturation and cytokine production when used alone. When used in combination with CD40L or GM-CSF, it potentiated/synergized the CD40L- or GM-CSF-induced effects.

4. Clinical trials
For several years, clinical trials in cancer patients have included drugs that interfere with inhibitory pathways of the immune system. The general aim is to de-block immune effector cells that can potentially help to control malignant diseases. Currently, there are clinically graded drugs available that block CTLA-4, PD-1, PD-L2 and LAG-3 [47]. The latter is IMP321, a soluble recombinant LAG-3–Ig fusion protein. IMP321 is used as immunological adjuvant for vaccination against infectious diseases or against cancer. Alternatively, IMP321 is used as monotherapy, or together with chemotherapy in cancer patients. Up to today, seven clinical studies have been or are being performed (Table 1).

The first two randomized Phase I trials consisted of treatment with IMP321 alone or IMP321 combined with Influenza or Hepatitis B surface antigens. Both studies revealed very good clinical tolerability with a low toxicity profile for the four dose levels of IMP321 (3, 10, 30 and 100 µg). In the first study (P001), the first arm (20 volunteers) showed that the four doses of IMP321 were safe, well tolerated and similar to placebo [48]. No specific anti-IMP321 antibodies were induced after a single subcutaneous injection. In the second arm (40 volunteers), most of the adverse events corresponded to systemic (asthenia, headache, vertigo, nausea) or local (pain and erythema) reactions commonly observed with influenza vaccines. Adding IMP321 to influenza vaccination did not increase the incidence or severity of adverse events. In the second study (P002), the combination of IMP321 with HBsAg was characterized by a good tolerability profile at the four doses tested [49]. A lower incidence of subjects experiencing adverse events was reported after injection of IMP321 or HBs antigen alone than after co-injection of Engerix-B, a vaccine containing alum-absorbed yeast-derived recombinant HBsAg. The most commonly observed non-serious adverse events included local injection site pain and erythema, as well as systemic symptoms such as nausea and headache. Injection site pains and erythema were considered certainly related to the study drugs, whereas nausea and headache were considered possibly related. Most of these adverse events were of mild to moderate intensity and resolved without any corrective treatment. With respect to the immunogenicity of the combination peptide and IMP321, the 10 and 30 µg doses have been shown to be the most effective in inducing antigen specific CD4⁺ T cells in the setting of an influenza vaccine, compared
with subjects vaccinated with influenza antigen only. Vaccination in the presence of IMP321 also showed a trend to induce an increased long-term memory CD4+ T cell subset. Similarly, the co-injection of IMP321 with HBs antigen resulted in an increased number of responders after a single injection, with more pronounced CD4+ and CD8+ T cell responses. Concerning CD4 T cell responses, injecting 10 or 30 µg IMP321 with 10 µg HBsAg without alum was equivalent to injecting 20 µg of alum-absorbed HBsAg (Engerix-B®) for the first injection, even though there was no depot effect in the former. For CD8+ T cell responses, only the groups injected with IMP321 contained high responders.

A study in metastatic melanoma patients (P006) is based on the rationale that vaccination with melanoma-derived antigens (short 9–10 amino-acid long peptides) may induce tumor-specific CD8+ T-cell responses, leading to objective tumor regression in a minority of these patients. Several adjuvants have been used with little success to date in increasing the percentage of responding patients as well as the level of CD8 T cell response. The design of study 006 was a Phase Ib/II clinical trial to test the safety and the immunostimulatory effect of IMP321 on CD8 T cell responses to a pool of 8 HLA-A*0201 binding peptides derived from the tumor antigens tyrosinase, gp100, and MAGE antigens. Patients were randomized to receive the peptides in saline, peptides admixed with Montanide (Incomplete Freund’s Adjuvants; IFA), peptides and IMP321, or peptides with IMP321 and Montanide. Vaccines are administered every 3 weeks. No adverse event related to IMP321 (5 injections, 0.5 mg) has been reported to date in the first 24 patients. Unfortunately, the study was halted due to regulatory problems with the peptide GMP-status. The data on disease-free survival at 3 years will be presented soon.

In study P007, HLA-A2 positive melanoma patients are receiving vaccinations based on the melanoma peptide Melan-A/MART-1, mixed together with Montanide (IFA), and IMP321 at two dose levels (25 or 250 µg), after lymphodepletion induced by short-term chemotherapy (fludarabine for 2 days, followed by cyclophosphamide for 3 days). The primary endpoints are toxicity and immune response. Preliminary results indicate that vaccination with IMP321 is well tolerated, and that IMP321 contributed significantly to the induction of immune responses, which were stronger as compared with historical control patients who underwent the same treatment but received vaccinations without IMP321. For this study, regulatory problems with peptides have been resolved; the study continues to recruit patients.

Monotherapy with IMP321 was done in a dose escalation study (P003) in patients with metastatic renal cancer. IMP321 was injected subcutaneously at doses of 50, 250, 1250, and 6250 µg every 2 weeks for a total of six times. The results showed good tolerance of the drug (no local toxicity, no major adverse events, and no dose-limiting toxicity). There was no induction of IMP321-specific antibodies, even at the highest dose. Lymphocyte activation was observed with an induction of both CD8+ T-cell and NK cell activation. CD8+

Table 1. Clinical trials with IMP321.

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<td>IMP22 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
<td>none</td>
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<td>IMP23 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
<td>none</td>
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<td>IMP24 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
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<td>IMP25 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
<td>none</td>
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<td>IMP26 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
<td>none</td>
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<td>IMP27 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
<td>none</td>
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<td>IMP28 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
<td>none</td>
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<td>IMP29 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
<td>none</td>
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<td>IMP30 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
<td>none</td>
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<td>IMP31 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
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<td>IMP32 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
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<td>IMP33 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
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<td>IMP34 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
<td>none</td>
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<td>IMP35 (Phase I, randomized)</td>
<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
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<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
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<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
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<td>Melan-A/MART-1, Tyrogp100, CEA, Melan-A peptide</td>
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<td>IMP46 (Phase I, randomized)</td>
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<td>IMP47 (Phase I, randomized)</td>
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<td>IMP48 (Phase I, randomized)</td>
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<td>None.</td>
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<td>IMP58 (Phase I, randomized)</td>
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</table>
T cell activation was sustained, and included long-lived CD28+ effector memory cytotoxic CD8+ T cells, in almost all patients treated with 6.25 and 30 mg IMP321. A reduction in tumor growth was observed in these patients. Progression-free survival was significantly better in patients receiving higher doses (> 6 mg) of IMP321: 7 out of 8 evaluable patients treated at the latter doses experienced stable disease at three months compared with only 3 out of 11 patients in the lower dose group (p = 0.015).

A study combining IMP321 with standard chemotherapy was performed in breast cancer patients [51]. In the absence of potent and registered breast cancer antigens today, an alternative therapeutic strategy is to amplify ‘natural’ pre-existing T cells responses. Chemotherapy induces tumor cell apoptosis leading to antigen release and T cell priming [52]. The rationale for associating immunotherapy and chemotherapy, the so-called chemoinmunotherapy approach, has been described in animal models but studies in humans with CpG failed to show clinical efficacy. However, enhancing chemotherapy-induced antigen release by giving a non-specific immunostimulatory factor that induces APCs to mature and transport tumor antigens to the local lymph nodes for presentation to T cells is an attractive therapy. This therapeutic approach is supported by preclinical studies that show encouraging results in the case of carcinomas [53-55]. The design of study P005 was as an open-label, non-randomized, dose escalation Phase I study for metastatic breast cancer patients. IMP321 was given at 250 μg and 1250, 6250 and 30,000 μg, combined with first-line chemotherapy consisting of weekly paclitaxel. The results of this study showed encouraging tumor regression that was significantly increased (p = 0.005) in the 30 patients of this study as compared with tumor regression in 2009 in three treatment groups, with 0, 3 and 6.5 mg IMP321 administered subcutaneously at the day after front-line doses of gemcitabine for 6 months, and a total of 12 injections. Endpoints are safety, immunogenicity and clinical results.

5. Expert opinion

Recent evidences show that antigen-specific T cells lack and/or loose critical functions that are essential for controlling infections or tumors, particularly at mid- and long-terms. Lack/loss of T cell function is accompanied by the expression of inhibitory receptors such as PD-1, CTLA-4, LAG-3, BTLA and further receptors [30]. Therapeutic approaches aiming at blocking the engagement of these inhibitory receptors provided encouraging results [56,57] and some biologicals are now being evaluated already in Phase III clinical trials [58]. Furthermore, blocking inhibitory receptors, in combination with active immunotherapy or chemotherapy, yields promising pre-clinical and clinical results [55]. Simultaneous blockade of multiple inhibitory pathways potentiates therapeutic effects and shows striking results in animal models [30].

The therapeutic activity of IMP321 (the human dimeric soluble form of LAG-3) is now well established in pre-clinical as well as clinical studies. It was recently shown that the combination of hLAG-3-Ig with chemotherapeutic agents is superior to either treatment on their own. Furthermore, a mouse model demonstrated that LAG-3 blockade may also be combined with blockade of other inhibitory receptors, such as PD-1, resulting in enhanced T cell activity and protection from disease. Further studies aiming at defining the mechanisms of synergy of such combined approaches will provide the rationale to select optimal combination therapies. Nevertheless, it is also necessary to continue basic research on the role of LAG-3 in vivo, and to identify the mechanisms of action of sLAG-3–Ig.

In conclusion, currently available data are encouraging and justify the further development of targeting of the LAG-3 pathway. In our experience, the start-up company Immuteq that produces IMP321 supports broad research in the field, and welcomes collaboration with industrial and academic partners. The availability of clinical-grade IMP321 for investigator-initiated clinical studies provides an unusually favorable basis for further rational development. In multiple Phase I/II studies that include precise analysis of biological and clinical responses, one can identify optimized treatment modalities and identify specifically responding patient populations. Subsequent Phase III trials can then focus on the most promising approaches.

Acknowledgments

We thank F Triebel (Immutep SA) for helpful discussion and reviewing the manuscript. We apologize for those scientists and clinicians whose studies could not be mentioned. We thank all members of our research groups, and all our collaborators for their dedicated contributions.

Declaration of interest

This work was supported by the Ludwig Institute for Cancer Research Ltd, the Cancer Research Institute (USA), the Cancer Vaccine Collaborative, Atlantic Philanthropies (USA), the Wilhelm Sander-Foundation (Germany), the Swiss Cancer League grant 02279-08-008, the Swiss National Science Foundation, and the Swiss National Center of Competence in Research (NCCR) Molecular Oncology.
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Affiliation
Sophie Sierro1 PhD, Pedro Romero1 MD & Daniel E Speiser2 MD
1Author for correspondence
1Ludwig Institute for Cancer Research Ltd CHEMIN DES BOVERESSES 155, 1066 EPALINGES, SWITZERLAND
2Lausanne University Hospital, Avenue Pierre-Decker 4, 1011 LAUSANNE, SWITZERLAND
Tel: +41 21 314 0182; Fax: +41 21 314 7477; E-mail: daniel.speiser@hopvd.ch