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T Lymphocytes infiltrating various tumour types express the MHC class II ligand lymphocyte activation gene-3 (*LAG-3*): role of LAG-3/MHC class II interactions in cell–cell contacts

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Abstract

The product of the *Lymphocyte Activation Gene-3* (*LAG-3*, CD223) is a high affinity MHC class II ligand expressed by activated $CD4^+$ and $CD8^+$ T cells, which can associate with the T cell receptor (TCR) and downregulate TCR signalling *in vitro*. We have also reported that a soluble mLAG-3Ig fusion protein works as a vaccine adjuvant *in vivo* in mice, enhancing Th1 and CD8 T cell responses. Here, we report that LAG-3 expression was found, using fluorescent activated cell sorting (FACS) analysis, on 11–48% of human tumour-infiltrating lymphocytes (TILs) isolated from eight freshly dissociated renal cell carcinomas (RCCs), and was restricted mostly to $CD8^+$ cells. Immunohistochemical analysis confirmed LAG-3 expression by TILs in 9/11 RCCs, as well as in tumours of different origins, such as melanomas (3/5) and lymphomas (7/7). Since not only antigen presenting cells (APCs), but also TILs themselves strongly express major histocompatibility complex (MHC) class II, we firstly investigated whether LAG-3/MHC class II T–T cell contacts might influence tumour cell recognition. However, cytotoxicity inhibition was not observed in two RCC-specific $CD8^+$ T cell clones in the presence of the LAG-3-specific MAb, and there was also no observed difference in the recognition of LAG-3-transfected or wild-type RCC by these cytotoxic T lymphocytes (CTLs). In contrast, MHC class II engagement by LAG-3Ig was found to enhance the capacity of immature dendritic cells to stimulate naive T cell proliferation and IL-12-dependent IFN-γ production by T cells *in vitro*. These results therefore provide support for a role for TIL-expressed LAG-3 in the engagement of class II molecules on APCs, thereby contributing to APC activation and Th1/Tc1 commitment, without downregulating cytotoxicity.

Keywords: MHC class II; T Lymphocytes; Dendritic cells; Melanoma; Renal cell carcinoma; Lymphoma

1. Introduction

Recent progress made in the understanding of anti-tumoral immunity has revealed that tumours of sufficient immunogenicity trigger an immune response in the host resulting in the recruitment of tumour antigen-specific T lymphocytes, which infiltrate the tumour tissue. Among these tumour-infiltrating lymphocytes (TILs), a variable proportion of CD8 T cells exerting cytotoxic functions against tumour cells has been observed [1–3]. Thus, tumour progression in cancer patients may not simply result from an absence of immune response, but rather from the inability of effector cells to control or destroy the tumour cells. Several mechanisms have been proposed to explain tumour resistance to destruction, including the production of immunosuppressive cytokines by the tumour cells, resistance of target cells to tumour necrosis factor alpha (TNFα) or Fas-mediated cytotoxicity [4], and the engagement of Killer Inhibitory Receptors (KIRs) on T cells by tumour MHC class I molecules [5]. In addition to T cells, dendritic cells (DCs), the most potent antigen presenting cells (APCs), have also been detected in tumour tissues [6,7]. In an attempt to develop efficient new strategies to cure cancer, characterisation of these players of antitumour cell immunity, and of how they

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interact and how their functions are impaired or may be boosted, is of critical importance.

The *Lymphocyte Activation Gene-3* (*LAG-3*), which is embedded in the *CD4* locus [8,9], encodes a protein, also termed CD223 (HLDA7 workshop), that is expressed in activated $CD4^+$ and $CD8^+$ T cells and associated with the CD3/T cell receptor (TCR) complex at the cell surface of these cells [10,11]. LAG-3 has structural similarities with CD4 [8] and like CD4 [12,13], binds major histocompatibility complex (MHC) class II molecules [14,15]. It may oligomerise at the cell surface to interact more efficiently with class II molecules [16]. Several findings indicate that LAG-3 expression participates in the type 1 (Th1/Tc1) T cell response. LAG-3 expression is associated with intracellular interferon-gamma (IFN-γ) production in both $CD4^+$ and $CD8^+$ subsets [17,18] and both IFN-γ and LAG-3 are upregulated by interleukin-12 (IL-12) [18]. In addition, both ectopically-expressed on murine tumour cells, and as a soluble fusion protein (mLAG-3Ig), LAG-3 has been shown to contribute *in vivo* to the commitment of murine T cells toward the Th1/Tc1 type, by cross-linking MHC class II molecules expressed on APCs [19,20]. *In vitro*, hLAG-3Ig-induced cross-linking of MHC class II molecules expressed by immature human DCs results in IL-12 secretion, a known Th1 inducer, without the need of a cell determinant 40 ligand (CD40L) signal [21].

However, we reported that LAG-3 participates in the cell–cell interactions between phorbol ester (PHA)-activated CD4 T cells which result in downregulation of their proliferation and cytokine production [15]. Moreover, cross-linking LAG-3 expressed by activated T cells with MAb prior to restimulation led to TCR signalling downmodulation and T cell unresponsiveness [10], indicating that LAG-3 expression by activated T cells renders them susceptible to as yet unclear MHC class II-dependent downregulation of their effector functions. These experiments also suggest that LAG-3 may indeed contribute to the induction of TIL unresponsiveness.

We examined here the possibility that TILs might express the LAG-3 antigen at their surface and that this could contribute to modulate their antitumour effector functions.

2. Materials and methods

2.1. Tumour samples, TILs and TIL-derived T cell clones

Tumour samples were obtained from patients undergoing surgical tumour ablation in our institution, and were used to prepare either frozen sections or single-cell suspensions and TIL cell lines as previously described in Ref. [23]. Generation of renal cell carcinoma (RCC) tumour-specific T cell clone 3B8 (TCR α/β^+ $CD4^-$ $CD8^+$, HLA-B7-restricted and specific for a nonameric intestinal carboxyl esterase epitope) from patient no. 8's TILs and clone 11C2 (TCR α/β^+ $CD4^-$ $CD8^+$, HLA-A2-restricted and specific for a decameric mutated HSP70-2 epitope) from patient no. 6's TILs were previously described in Refs. [3,24].

2.2. Fluorescent activated cell sorting (FACS) analysis of TIL suspensions

LAG-3 expression on TILs was assessed by direct immunofluorescence using the fluorescein isothiocyanate (FITC)-coupled LAG-3-specific 17B4 MAb recognising the LAG-3.1 epitope [25]. Additional staining with anti-CD3 (PerCP), or anti-CD4 (phycoerythrin (PE)) or anti-CD8 (PE) allowed a gate analysis of total T cells and T cell subsets, respectively. Freshly dissociated single cell suspensions of RCC TILs were incubated with antibodies for 30 min and washed twice in saline buffer. Inhibition experiments consisted of preincubation of 17B4 with a specific 208b peptide or a control tetanus toxoid (TT) peptide at varying concentrations prior to incubation with TILs from patient no. 1, followed by FACS analysis.

2.3. Immunohistochemical analysis of frozen tissue sections

The expression of LAG-3 protein was studied by immunostaining seven lymphomas, five melanomas and 11 RCCs. Among the RCCs, six were primary tumours and five were metastatic. Five-micron-thick frozen sections were prepared for all samples. The sections were fixed in acetone for 10 min at room temperature, air-dried, and stored at $-20^\circ C$. Before use, the slides were fixed in acetone for 5 min and air-dried. After a blocking step in 5% non-fat milk in Tris/NaCl buffer pH 7.6 (TBS), the sections were incubated for 1 h at room temperature with primary MAbs at relevant dilutions in TBS: 1/50 for CD3, CD4 and CD8; 1/100 for TiA-1 (T-cell intracellular cytotoxic protein, Coulter, Hialeah, FL, USA); and 1/150 for LAG-3 (20 μg/ml purified 17B4 MAb, IgG1), after a dilution test on reactive lymph node frozen sections. Mouse MAbs were then recognised by a sequential incubation with rabbit anti-mouse immunoglobulins (Z259 Dako SA), followed by a mouse antibody conjugated to an alkaline phosphatase anti-alkaline phosphatase complex (APAAP) (D651, Dako SA), and were revealed by Naphtol-Fast Red (Sigma) and counterstained with haematoxylin [26].

2.4. Genetic constructs and transfection of tumour cells with LAG-3

To generate soluble hLAG-3 molecules, the extracellular domains of hLAG-3 were fused to the hIgG1

Fc portion, as previously described in Ref. [15]. The resultant recombinant proteins were produced in CHO cells. Batches of feedstock were thawed and sLAG-3Ig was captured using cation-exchange chromatography (Carboxy Sulfon column). Following this, partially purified sLAG-3Ig was subjected to metal chelate affinity chromatography (IMAC), to generate material with a purity >90% (gel electrophoresis, SE-high performance liquid chromatography (HPLC)). Purified material was concentrated to 5 mg/ml (in phosphate buffer) and aliquots were stored at −80°C (Dr M. Subramanyam and M. Tepper, Ares Advanced Technology, MA, USA). The amount of any potential contamination of the purified protein with bacterial endotoxin (LPS) was determined using the chromogenic *Limulus amoebocyte* lysate assay (BioWhittaker, Walkerville, MD, USA), which measures the activity of a *L. amoebocyte* protease following activation by LPS. A calibration curve of enzymatic activity versus LPS was constructed to determine endotoxin units (EU) in the test sample. Values of less than 4 EU/mg were obtained for hLAG-3Ig.

The RCC cell line from patient no. 6 was transfected with cDNA encoding *LAG-3* as previously described in Ref. [19]. Briefly, cells were double electroporated in the presence of cDNA. Cells were then cultured in complete medium containing Hygromycin B at 200 μg/ml (Sigma), and cloned by limiting dilution. LAG-3-expressing cells were selected by FACS analysis using the LAG-3-specific 17B4 MAb.

2.5. Cytotoxicity assay

T cell clone 3B8 (3) derived from patient no. 8's TILs was incubated (10^6 cells/ml) with a 5-fold saturating concentration of LAG-3-specific 17B4 (5 μg/ml) or control antibody (NKTa) for 1 h prior to being added to 5000 syngeneic ^{51}Cr-labelled tumour cells at varying effector/target (E/T) ratios (0.5–20 T cells per target cell corresponding to 10^5–2500 T cells per point) in 96 flat-bottomed wells in a final volume of 200 μl. Supernatants were harvested after 4 h and assessed for radioactivity. Alternatively, 5000 tumour cells were added, the supernatant collected after 18 h, and the TNF content was determined by testing its cytotoxic effect on WEHI-164 13 cells [10]. The WEHI cells are susceptible to lysis by both TNF-α and TNF-β. Because T cells produce both cytokines, the test measures the combined effect of the two cytokines. Results were expressed relative to amounts of recombinant TNF-β leading to similar cell death as assessed in the colorimetric assay. T cell clone 11C2 [24], derived from patient no. 6's TILs, was cultured with LAG-3-expressing or wild type syngeneic ^{51}Cr labelled tumour cells for 4 h, and the amount of chromium released was assayed.

2.6. Dendritic cell preparation and allogeneic T cell stimulation

Human immature dendritic cells were prepared as described in Ref. [27], except that peripheral blood monocytes were obtained by cold aggregation [28] and were depleted of T cells by rosetting on AET-treated sheep red blood cells. After additional selection by adherence [27], cells were cultured in complete medium containing GM-CSF (25 ng/ml) and IL-4 (25 ng/ml) for 7 days [21]. Recombinant granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4 were provided by D. Bron (Bordet Institute, Brussels, Belgium) and Immunex Corp. (Seattle, WA, USA), respectively.

For allogeneic MLR-responding cells, either adult or neonatal (umbilical cord blood) T cells were highly purified by rosetting of the monocyte-depleted peripheral blood mononuclear cells (PBMCs) followed by Lympho-Kwik T treatment (One Lambda, Canoga Park, CA, USA). These T cells (10^6/ml) were then stimulated with different numbers of irradiated (55 Gy) immature DCs in complete HB101 medium (Irvine, Santa Ana, CA, USA) containing 10 μg/ml Polymyxin B (an inhibitor of the lipopolysaccharide (LPS)-induced monocyte response; Sigma, St. Louis, MO, USA). Either hLAG-3Ig or normal human IgG as a negative control (both at 5 mg/ml) were added to cultures, and DNA replication was measured by addition of [^{3}H]-Thymidine (1 μCi/well) on day 3 for 16 h. Culture supernatant was collected on day 3 and the IFN-γ content was determined using a previously described radioimmunoassay [28]. The goat anti-human IL-12 Ab was a generous gift from M. Gately (Hoffmann LaRoche, Nutley, NJ, USA).

3. Results

3.1. FACS analysis of RCC-derived TILs

We have previously described the expression of LAG-3 on TILs from 1 RCC patient [23]. We then became interested in assessing whether this observation was an exception or a common feature of most RCC TILs. 8 patients' tumours were therefore enzymatically dissociated and their LAG-3 expression analysed by FACS. As shown in Fig. 1 and summarised in Table 1, all RCC patients had LAG-3-expressing TILs, although the expression varied from 11 to 48%. LAG-3 expression is often quite low, and therefore difficult to detect. In order to ensure the specificity of anti-LAG-3 antibody recognition, we preincubated the antibody with a peptide corresponding to its specific epitope (208b) or a control peptide (for the tetanus toxoid, TT) prior to FACS analysis. As shown in Fig. 1b, we

observed a dose-dependent inhibition of antibody recognition when the antibody was coincubated with the peptide 208b, but not when incubated with control TT peptide, demonstrating the specificity of antibody recognition. In contrast to the constant expression of LAG-3 on a subset of TILs, TILs do not express the activation antigens CTLA-4 and 4-1BB (data not shown).

As LAG-3 has been described on T CD4 cells and CD8 cells activated *in vitro*, we also analysed the expression of LAG-3 on these two TIL subpopulations using two-colour FACS analysis. As shown in Fig. 2, only $CD8^+$, and not $CD4^+$ TILs, were shown to express LAG-3.

3.2. LAG-3 expression on TILs in frozen tissue sections

Using immunohistochemistry on frozen tissue sections (paraffin-embedded tissue sections are not stained with either 11E3 and/or the 17B4 LAG-3-specific MAbs) (25), $LAG\text{-}3^+$ cells were identified in 3 out of 5 melanomas, 9 out of 11 RCC and 7 out of 7 non-Hodgkin's lymphomas (Table 2). Such cells were usually rare and scattered throughout the tumour mass, in close contact with the tumour cells, as shown in Fig. 3 in the case of a lymphoma. The frequency of the LAG-3-expressing cells can then be compared with that obtained with the TiA-1 molecule, a marker associated with the cytotoxic function of CD8 T cells. Finally,

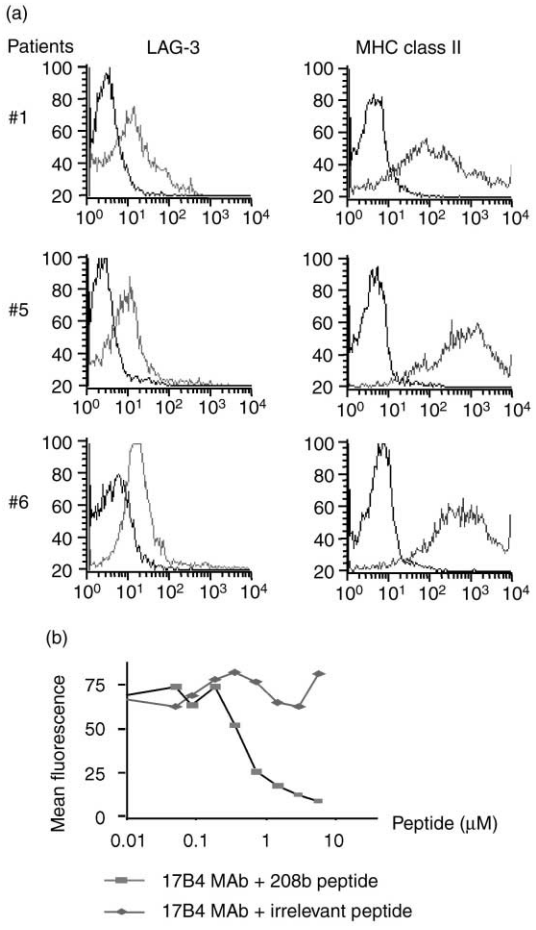

Fig. 1. Tumour infiltrating lymphocytes (TILs) express lymphocyte activation gene-3 (LAG-3). (a) A triple staining analysis of TILs: TILs were triple-stained with anti-CD3 (PerCP), anti-LAG-3 (17B4; fluorescein isothiocyanate (FITC)) and anti-major histocompatibility complex (MHC) Class II molecules (phycoerythrin (PE)). Shown are the LAG-3 and MHC II profiles of $CD3^+$-gated cells for 3 patients, which are representative of the 8 patients studied. (b) Specific inhibition of 17B4 staining. The LAG-3-specific MAb was preincubated with a specific peptide epitope (208b) or a control epitope (TT) at different molarities prior to staining of TILs from patient no. 1. Stained cells were then analysed by fluorescent activated cell sorting (FACS).

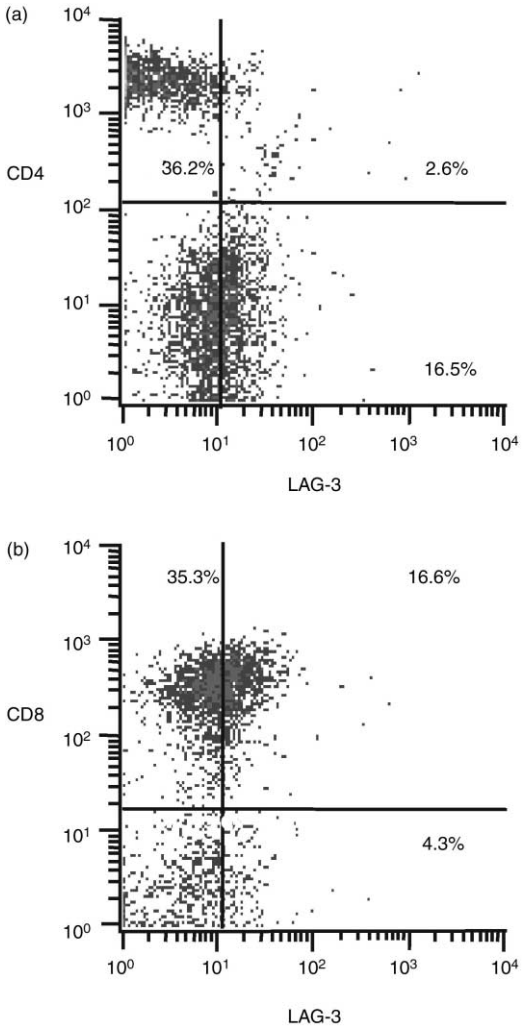

Fig. 2. Expression of lymphocite activation gene-3 (LAG-3) on $CD8^+$ and $CD4^+$ subpopulations of tumour infiltrating lymphocytes (TILs). TILs were analysed by triple-staining with anti-CD3 (PerCP), anti-LAG-3 (17B4; fluorescein isothiocyanate (FITC)) and (a) anti-CD4 or (b) -CD8 (phycoerythrin (PE)). LAG-3 and CD4 or CD8 expression of $CD3^+$-gated cells ($n = 5000$) from patient no. 7 are shown and values indicate percentages in each quadrant. This experiment was reproduced using TILs from patient no. 2, with identical results.

Table 1
Expression of LAG-3 and MHC class II molecules on $CD3^+$ TILs

Patient no.	% positive cells		
	$CD3^+$/LAG-3^+	$CD3^+$/MHC class II^+	$CD3^+$
1	48	89	89
2	41	65	85
3	11	76	86
4	16	81	85
5	31	92	96
6	32	83	88
7	20	ND	96
8	19	89	91

LAG-3, lymphocyte activation gene-3; MHC, major histocompatibility complex; TILs, tumour infiltrating lymphocites; ND, not determined. Single-cell suspensions of renal cell carcinoma (RCC) patients' TILs were stained as described in Materials and methods and the lymphocyte population analysed for LAG-3 and MHC class II expression.

LAG-3^+ cells were also found in the tumour stroma surrounding the beds of carcinoma cells in the majority of RCCs and melanomas studied (data not shown).

3.3. Recognition of RCC tumour cell lines by specific cytotoxic T lymphocytes (CTLs) is not modulated by LAG-3/MHC class II interactions

We have previously presented evidence that LAG-3/MHC class II interactions play an inhibitory role in T–T cell contacts of $CD4^+$ T cell clones [15], and that LAG-3 cross-linking induces T cell unresponsiveness to further TCR/CD3 engagement [10]. We therefore sought to investigate whether LAG-3/MHC class II interactions could have a modulatory effect on *in vitro* functions of $CD8^+$ T cell clones derived from TILs. T cell clone 3B8, derived from RCC patient no. 8, was incubated with the 17B4 LAG-3-specific MAb prior to the addition of syngeneic antigen-presenting tumour cells. As shown in Fig. 4, the blocking of LAG-3/MHC class II interactions between T cell clones had no effect on $CD8^+$ T cell responses (cytotoxicity and TNF-β secretion) to tumour cell antigen.

To assess the effect of MHC class II cross-linking on these $CD8^+$ T cells caused by LAG-3 during TCR-mediated antigen recognition, we transfected the tumour cell line from patient no. 6 with *hLAG-3* cDNA. As shown in Fig. 5a, these cells stably expressed the LAG-3 protein and thus can engage MHC II molecules borne by the T cells they stimulate. However, the presence of LAG-3 on these tumour cells did not modulate the cytotoxic capacity of syngeneic tumour-specific T cell clone 11C2 (Fig. 5b). Altogether, these results suggest that both LAG-3 and MHC class II molecules, when expressed on $CD8^+$ T cell clones derived from TILs, do not provide inhibitory signals when engaging their respective ligands.

Table 2
LAG-3^+ cells were detected in most tumour samples using immunohistochemical analysis

Case no.	Diagnosis	Antibodies				
		CD3	CD4	CD8	TiA-1	LAG-3
1	Melanoma	±	++	++	+	+
2	Melanoma	+	+	+	–	–
3	Melanoma	+	++	+	–	+
4	Melanoma	++	++	++	–	+
5	Melanoma	++	++	++	–	–
6	RCC	++	++	++	++	+
7	RCC[a]	++	++	++	++	+
8	RCC[a]	+	++	+	+	+
9	RCC[a]	++	++	++	++	++
10	RCC[a]	++	++	+	+	+
11	RCC	+	+	–	–	–
12	RCC[a]	+	+	+	+	+
13	RCC	++	++	++	++	++
14	RCC	++	++	+	+	–
15	RCC	+	+	+	+	+
16	RCC	+	++	++	+	+
17	Follic. lymphoma	+++	++	++	++	+
18	DLBCL	+++	+++	++	++	++
19	DLBCL	+++	+++	++	+	+
20	DLBCL	+++	+++	++	++	+
21	PTCL	+++	+++	++	++	+
22	PTCL	++	+++	++	+	++
23	PTCL	+++	+	+	+++	+

DLBCL, diffuse large B cell lymphoma; PTCL, peripheral T cell lymphoma; TiA-1, T-cell intracellular cytotoxic protein; LAG-3, lymphocyte activation gene; RCC, renal cell carcinomas.

The RCC patients (nos. 6–16) are different from the series shown in Table 1. Number of positive cells: (–) absence, (+) <10 cells per field (objective ×25); (++) 10–50 cells; (+++) >50 cells.

[a] Metastasis.

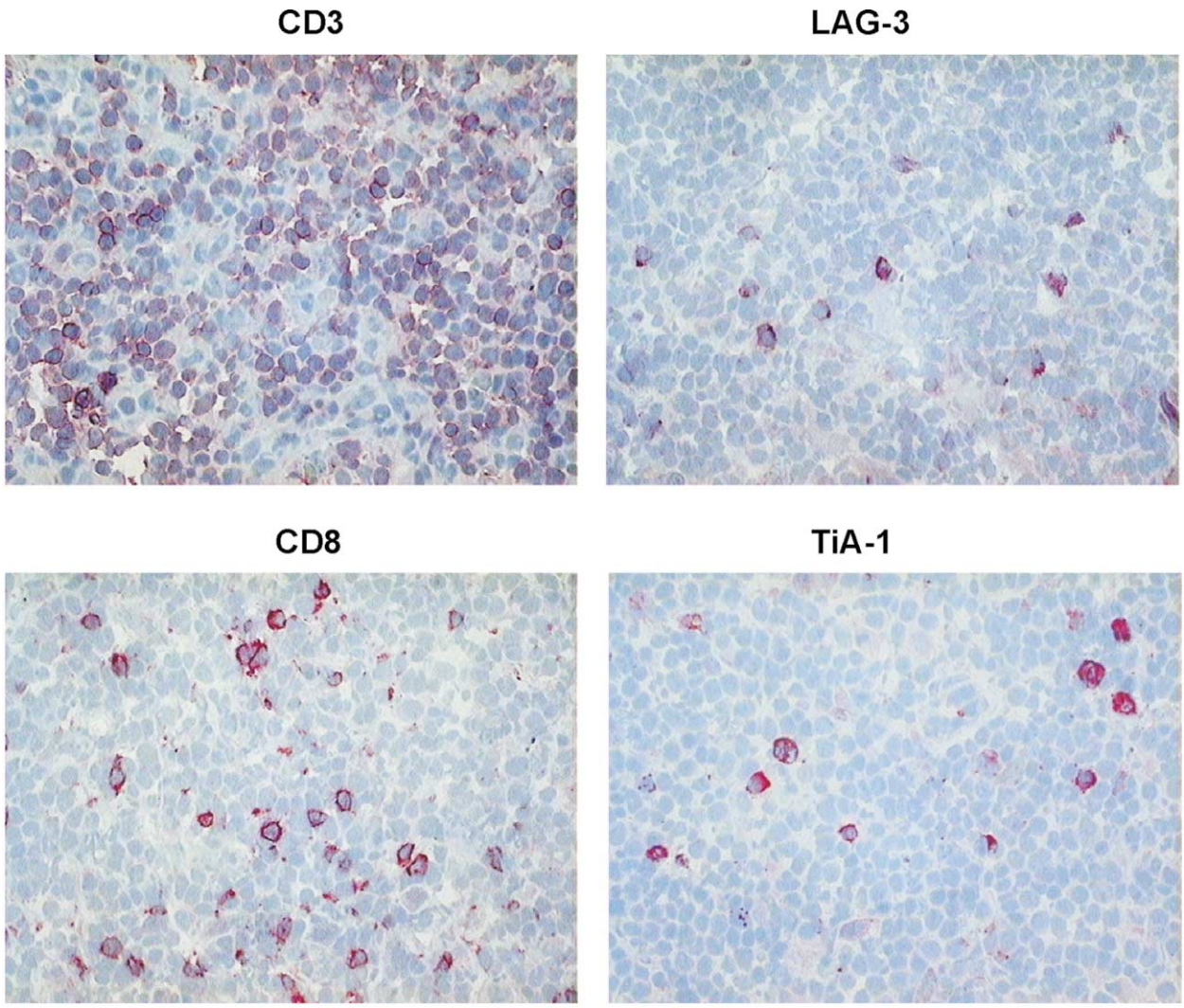

Fig. 3. Immunohistochemical analysis of CD3, CD8, T-cell intracellular cytotoxic protein (TiA-1) and lymphocyte activation gene-3 (LAG-3) in a frozen tissue section of a lymphoma. Slides were immunostained using APAAP, and counterstained with haematoxylin as described in Materials and methods. In the case of a diffuse large B-cell lymphoma (case no. 18 in Table 2), a large number of $CD3^+$ T cells were evenly distributed within the tumour cells. A proportion of the T cells were $CD8^+$ and expressed the TiA-1 marker for cytotoxicity. LAG-3^+ cells were detected with an overall distribution similar to that of the cytotoxic cells. Similar results were obtained with the renal cell carcinomas (RCC) and melanoma tumours.

3.4. LAG-3-stimulation increases the capacity of immature DCs to induce T-cell proliferation and IFN-γ production

We have recently shown that T cells expressing LAG-3 can interact in a TCR-independent fashion with MHC class II-expressing monocytes to induce IFN-γ and IL-12 production [21]. Furthermore, LAG-3Ig interactions with MHC class II-expressing DCs lead to TNF-α and IL-12 production [21]. In order to examine whether MHC II engagement by LAG-3 might also increase the stimulatory capacities of immature DCs, we firstly generated monocyte-derived immature DCs *in vitro* by culturing human blood monocytes in medium supplemented with GM-CSF and IL-4. These DCs were then used to stimulate purified T cells from adult peripheral blood or umbillical cord blood (i.e. naïve MHC II^-, LAG-3^- T cells) in an allogeneic mixed-lymphocyte reaction (MLR), in the presence of hLAG-3Ig or normal human IgG as the control. We observed that LAG-3-stimulated immature DCs have a 4- to 5-fold increased capacity to stimulate adult or naive T cell proliferation (Fig. 6a), and an approximately 2-fold capacity to upregulate IL-12-dependent IFN-γ production (Fig. 6b).

4. Discussion

In this present study, we strengthen and also expand on our previous observation that a subset of TILs express the LAG-3 antigen (CD223), a high affinity MHC class II ligand [14,29]. We had previously reported that LAG-3 was expressed on a subset of TILs

derived from enzymatically-dissociated RCCs in 1 patient [23]. Suspecting that LAG-3 was not detected in all analysed cases due to technical reasons, such as tumour dissociation and the staining procedure used, we further examined LAG-3 expression using both immunocytometric analysis of freshly dissociated single-cell suspensions and immunohistochemistry of frozen tissue sections.

Firstly, using FACS, we observed a heterogenous, but constant expression of LAG-3 on $CD8^+$ TILs of all 8 patients studied. This expression is specific, as it is inhibited by preincubation of the hLAG-3 MAb with its specific peptide epitope. LAG-3 was found exclusively on $CD8^+$, and not on $CD4^+$, TILs. Indeed, we have previously reported that although both CD4 and CD8 T cells can express the LAG-3 molecule *in vitro*, CD8 T cells have a higher capacity to express LAG-3 than CD4 T cells [18,25]. The higher capacity of $CD8^+$ versus $CD4^+$ T cells to express this MHC class II ligand upon stimulation, both *in vitro* and *in vivo*, may result from a different regulation of *LAG-3* expression in the two T cell types. We have previously shown that the *LAG-3*

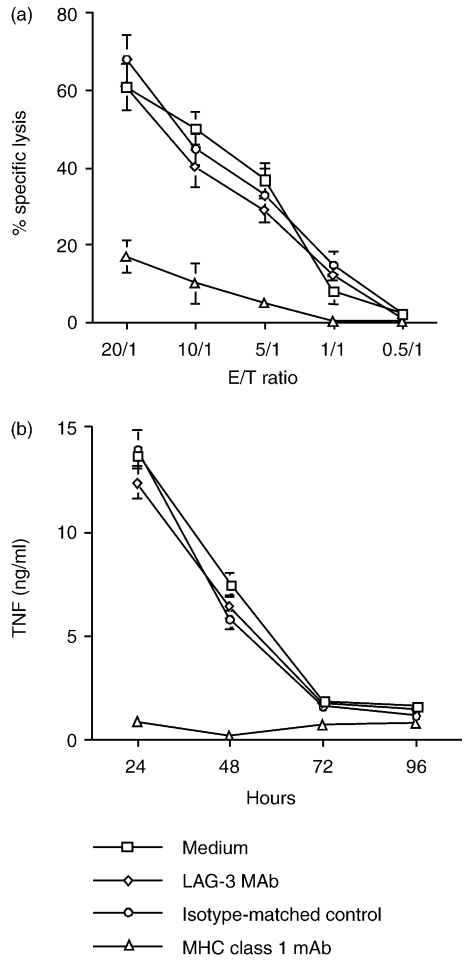

Fig. 4. Recognition of syngeneic tumour cells by renal cell carcinomas (RCC)-specific T cell clones are not modified by lymphocite activation gene-3 (LAG-3)/major histocompatibility complex (MHC) class II interactions. (a) Cells from the 3B8 T cell clone were preincubated with either LAG-3-specific MAb (17B4, 5 μg/million cells) or control MAb (NKTa, same dose) prior to culturing for 4 h with 5000 ^{51}Cr-labelled tumour cells at indicated ratios: 20/1–0.5/1 corresponding to 10^5–2500 T cells. The addition of MHC class I-specific MAb (10 μg/ml) serves as a control for lysis specificity. At the end of culturing, the radioactivity released in supernatants was counted. Results are shown as the mean of triplicate wells (±standard deviation (S.D.)) and are representative of two independent experiments undertaken with clones derived from patient nos. 6 (11C2 T cell clone) and 8 (3B8 clone). (b) 3B8 T cell clone (10^6 cells/ml) was preincubated with LAG-3-specific MAb (17B4) or control MAb (NKTa) prior to addition of 5000 tumour cells. The production of tumour necrosis factor (TNF) was assessed through its cytotoxic effect on WEHI cells 18 h later.

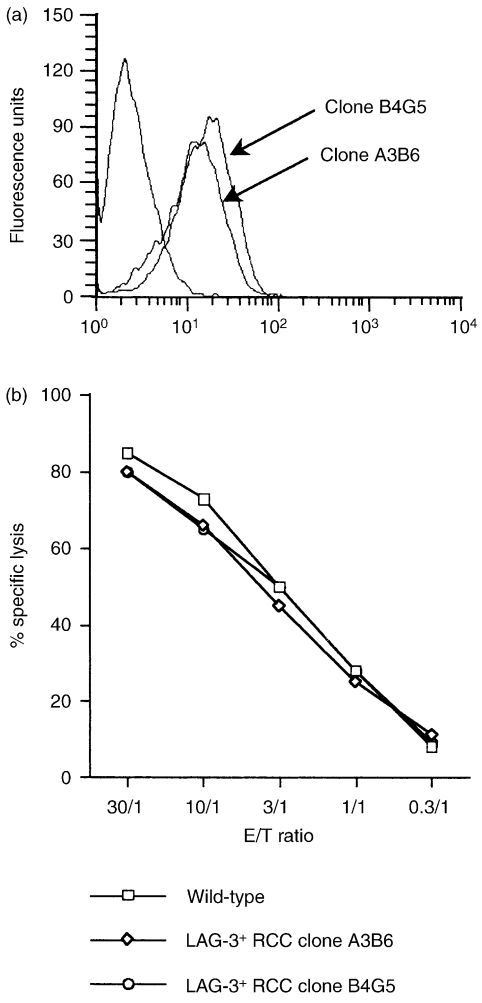

Fig. 5. Cytotoxicity of renal cell carcinomas (RCC)-specific T cell clones on lymphocyte activation gene-3 (LAG-3)-transfected syngeneic tumour cells is not modified compared with wild-type targets. (a) FACS analysis of *LAG-3*-transfected tumour cells. *LAG-3*-transfected tumour cell clones (A3B6 and B4G5) from patient no. 6 were analysed by fluorescent activated cell sorting (FACS) using LAG-3-specific MAb 17B4. Shown are the profiles of the two LAG-3^+ RCC clones (arrows) stained with 17B4 compared with the wild-type RCC cell line. (b) T cell clone 11C2 (patient no. 6) was incubated with 5000 ^{51}Cr-labelled tumour cell clones at varying effector/target ratios, and [^{51}Cr] release assayed after 4 h incubation. Results are representative of two independent experiments.

promoter is embedded in the *CD4* locus [9] between two *CD4* enhancer-like regions that control CD4 expression in the mouse and that this promoter can be partially silenced in *in vitro* experiments [18]. Higher LAG-3 expression is observed on recently activated T cells [25] compared with TILs (the present work) suggesting that TILs are in a late activation phase after triggering. Indeed, they expressed high levels of the late activation antigen MHC class II. Following this view, LAG-3 would be downregulated, but still detectable on $CD8^+$ T cells whereas it would have disappeared from $CD4^+$ T cells. As an alternative explanation, as has been proposed to explain the inefficiency of the TILs to fight the cancer [4], the tumour cells could contribute to deactivate the TILs, resulting in a reduced expression of the LAG-3 Ag. The cytokine context in which TILs are stimulated and the cytokines found in tumours are still poorly understood. Considering the capacity of LAG-3 to participate in DC-T interactions and favour a Th1 response, any therapeutic attempt to reactivate the TILs and among other effects to restore LAG-3 expression appears desirable.

Secondly, immunohistochemical staining of frozen tumour sections allowed us to examine LAG-3 expression and the location of $LAG\text{-}3^+$ TILs in the tumours, as well as to compare the expression of LAG-3 on RCC, melanoma and lymphoma tumours. We observed that a subset of TILs of either tumour type expresses LAG-3. $LAG\text{-}3^+$ TILs were scattered all over the tissue sections, and these close contacts with tumour cells were more obvious in B and T non-Hodgkin's lymphomas. They were also detected in the tumour stroma in most RCC cases and melanomas studied, as well as in the peritumoral tissue where they could interact with cells implicated in the immune response, which also share a similar distribution (e.g. dendritic cells) [30]. The observation that LAG-3 is expressed on TILs from different tumour histotypes suggests that it could be a general feature of T cells involved in an unsuccessful immune response, rather than a direct effect of certain tumour cells on T cells.

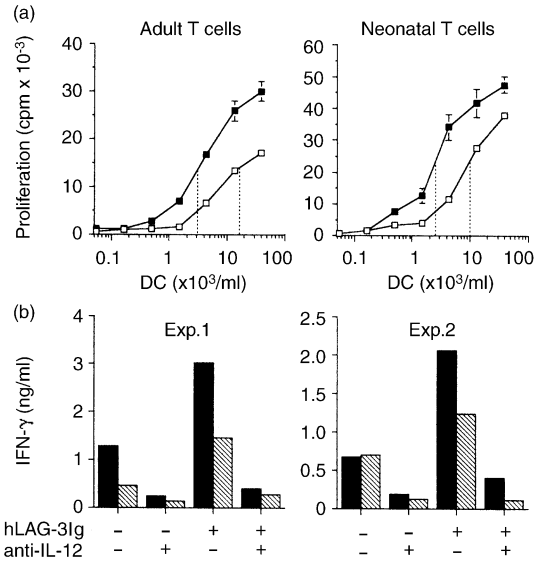

Fig. 6. Lymphocyte activation gene-3 (LAG-3)Ig stimulation of immature dendritic cells enhances their allostimulatory capacity. (a) Various numbers of immature DCs were used to stimulate adult or neonatal peripheral blood allogeneic T cells (10^6 cells/ml) in the presence (closed symbols) or absence (open symbols) of hLAG-3Ig (2 μg/ml) for 3 days. Shown is the mean±standard deviation (S.D.) of triplicates in one experiment, which is representative of three independent experiments undertaken (cpm, counts per minute). (b) Allogeneic adult T cells were stimulated with immature DCs at two different doses (40×10^3/ml, filled bars, or 13×10^3/ml, hatched bars) in the presence or absence of hLAG-3Ig (2 μg/ml) and either goat anti-interleukin (IL)-12 (10 μg/ml) or goat normal IgG. The supernatant was collected after 3 days for interferon-gamma (IFN-γ) measurement. The results of two independent experiments are shown.

A possible role for LAG-3 late after T cell activation could be to downregulate T cell effector functions, as suggested by our observation that LAG-3 cross-linking on activated T cells renders them unresponsive to an optimal TCR-mediated stimulation [10], and that T–T interactions between PHA-activated CD4 T cells transmit inhibitory signals involving LAG-3 [15,22]. Whereas RCC tumours are MHC class II^- targets unable to engage LAG-3, simultaneous expression of MHC class II molecules and of LAG-3 suggests that T–T contacts might contribute to TIL deactivation. We followed this line of reasoning in two ways: firstly, by using our blocking anti-LAG-3 Ab 17B4 to block the cytotoxicity of a TIL-derived CD8 T cell clone against its target tumour, and secondly, by targeting CTLs to a LAG-3 transfected syngeneic RCC tumour cell line. Both experiments led us to observe that blocking or enhancing LAG-3/MHC class II contacts on T cells failed to affect, either positively or negatively, their examined effector functions (i.e. cytotoxicity and TNF release). In our previous work [22], blocking LAG-3 function resulted in an enhancement of $CD4^+$ T cell proliferation and of cytokine production, revealing the existence of an inhibitory pathway. In the present work, the inability of the same Ab to affect the cytoxicity of $CD8^+$ TILs indicates that either helper (CD4) and cytotoxic (CD8) functions are regulated differently, or that the T–T interaction involving LAG-3 affects $CD4^+$ and $CD8^+$ T cells differently. In any event, this result demonstrates that the presence of LAG-3 on TILs may not be the cause of the immunosuppression often observed in TIL subpopulations.

RCC tumours stimulate a strong immune response in the host, resulting in a massive infiltration of the tumour mass by immune cells. Although tumour cells display MHC I molecules, the lack of costimulatory molecules generally renders them poorly immunostimulatory, and it has recently been understood that this antitumour immune response is triggered by profes-

sional APCs, such as dendritic cells, which capture and present tumour-derived molecules. Dendritic cells not only recruit MHC class II-restricted $CD4^+$ T cells, but also MHC class I-restricted $CD8^+$ T cells which become the cytotoxic T effectors targeted towards the tumour cells. Whether TILs interact with dendritic cells in the tumour tissue is not known. Infiltrated DCs have been observed in close association with tumour cells in RCCs [6,7], and recently Banchereau and colleagues [30] reported that T cells (a majority of $CD8^+$) infiltrating breast cancer tumours were often found associated with DCs expressing a mature phenotype. Due to the fact that mature DCs are generally found in lymphoid organs, the authors suggested that an ongoing immune response was possibly taking place there. Following the hypothesis that DCs could interact with TILs in solid tumours, LAG-3 molecules expressed by TILs may engage MHC class II molecules on DCs and play a role in such contacts.

The interaction between DCs and T cells is a bidirectional exchange of signals which can result in activation and maturation [31]. Naive T cells do not express LAG-3 and have a weak capacity to activate DCs [32]. In order to evaluate the role played by LAG-3 in T cell–DC interactions, we tested the ability of LAG-3, used in a soluble form, to compensate for the absence of membrane LAG-3 on naive T cells interacting with immature DCs. We report here that the signal given by sLAG-3 indeed increases the capacity of immature DCs to induce naive T cell proliferation by 4–5 times, indicating that the DCs have undergone a functional maturation. Moreover, we show that IL-12-dependent IFN-γ production, a key event for the development of a cell-mediated Th1 response, is also enhanced 2-fold in LAG-3-stimulated cultures. This expands on our previous finding that sLAG-3 stimulates DCs [21], and provides a likely mechanism for explaining the mLAG-3Ig-adjuvant effect observed *in vivo* in mice [20]. Soluble LAG-3 has been successfully used to boost the immune response directed against tumours in the mouse [19], showing the potential clinical interest of such reagents exerting an adjuvant role in a tumour vaccine.

Altogether, these observations strongly suggest that LAG-3 expression by activated T cells may play a beneficial role in *in situ* T cell–DC interactions, and therefore favours the expression of the Th1-type response to target tumours.

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