A Soluble Form of Lymphocyte Activation Gene-3 (IMP321) Induces Activation of a Large Range of Human Effector Cytotoxic Cells

Chrstelle Brignone, Caroline Grygar, Manon Marcu, Knut Schäkel and Frédéric Triebel

*J Immunol* 2007; 179:4202-4211; doi: 10.4049/jimmunol.179.6.4202

http://www.jimmunol.org/content/179/6/4202

References

This article cites 39 articles, 20 of which you can access for free at: http://www.jimmunol.org/content/179/6/4202.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
A Soluble Form of Lymphocyte Activation Gene-3 (IMP321) Induces Activation of a Large Range of Human Effector Cytotoxic Cells

Chrystelle Brignone,* Caroline Grygar,*, Manon Marcu,*, Knut Schaakel,† and Frédéric Triebel1*


A n efficient immune response against cancer cells requires the activation of type 1 cytotoxic T cells (Tc1),2 NK cells, and APC through appropriate cytokines/chemokines. Coordinated innate and acquired immune responses are required and many secreted and cell-associated factors have been recognized as important mediators that regulate and link these two arms of host defense. A major aim of cancer immunotherapy research is to find immunopotentiators able to prime APC to direct this cytotoxic effector cell-dominated response against tumor.

In addition to innate immunity ligands (e.g., TLR agonists), the immune response involves two adaptive immunity ligands that are expressed on activated T cells and bind to non-TLRs expressed on dendritic cells (DC). These are the CD40L and lymphocyte activation gene-3 (LAG-3 or CD223) human proteins. LAG-3/MHC class II interaction, together with CD40L/CD40 interaction, is involved in IL-12 production by APC in vitro (1). Recombinant soluble dimeric LAG-3 (LAG-3lg), like sCD40L, can directly induce in vitro-differentiated DC to produce Th1 cytokines or chemokines such as CCL22 and CCL17 which are known to direct the migration of maturing DC to lymph nodes (2, 3), whereas TLR agonists preferentially induce inflammatory cytokines/chemokines (e.g., IL-1β, IL-8) (3). On immature human monocyte-derived DC, LAG-3lg binds to a restricted subset of MHC class II molecules located in membrane lipid raft microdomains (2, 4) which represents ~15–20% of all MHC class II molecules on immature DC (5). This MHC class II subset is required for the induction of CD8+ T cell responses to exogenous Ag (i.e., cross-presentation) by inducing DC to process the Ag for class I presentation (6). This membrane compartmentalization of MHC class II for LAG-3-induced DC licensing is also important for the induction of the DC-induced Th1 phenotype (7).

In vivo, engagement of MHC class II by the LAG-3lg molecule coinjected with an Ag induces DC to mature and migrate to lymph nodes followed by the priming of naive CD4+ Th cells and the cross-priming of CD8+ CTLs (2, 3, 8). In mice, tumor rejection induced by LAG-3 was associated with induction of tumor-specific CD8 cells (9, 10) and presence of CD86-expressing APCs, NK cells, granulocytes, and infiltrating T cells as well as production of IFN-γ, CCL2/MCP-1, and CCL5/RANTES at the tumor site (11). In humans, LAG-3lg stimulation of the PBMCs of both cancer patients and healthy donors in the presence of 9-mer peptides can boost CD8+ T cell memory response or prime naive T cells in vitro (12). The peptide-specific T cells generated in the presence of LAG-3lg were endowed with specific cytotoxic activity against the relevant tumor cells and enhanced release of type 1 cytokines (12).

A natural soluble form of LAG-3 exists also as a serum protein (sLAG-3) (13) and there are some indications that sLAG-3-associated cell-mediated mechanisms such as Th1/Tc1 responses do have an impact on resistance to disease and on survival. The concentration of serum sLAG-3 is inversely correlated with that of sCD30, a well-established Th2 marker (14). In large series of
patients, high levels of serum sLAG-3 have been shown to be associated with resistance to tuberculosis, a disease where Th1 responses are crucial in defense against Mycobacteria (15). Finally, studying patients’ sera collected at the time of first diagnosis for breast cancer, we found that both disease-free and overall survival rates at 10 years were higher in patients who had higher levels of sLAG-3 at diagnosis (16).

Together, these data induced us to start evaluating the safety and the clinical and biological effects of this first-in-class product, a clinical-grade human LAG-3Ig fusion protein termed IMP321 (17). In this study, we report on the results of ex vivo short-term experiments where IMP321 recruited and activated a large range of effector cells in both innate and acquired immune responses.

Materials and Methods

Blood collection and PBMC isolation

Blood samples from healthy volunteers and metastatic cancer patients (6 breast and 15 renal carcinomas) with informed consent were collected in heparinized lithium tubes (Vacutainer; BD Biosciences) or were obtained from buffy coats provided by EFS. PBMCs were isolated by Ficoll-Paque density gradient (Pharmacia) using LeucoSep tubes (Greiner Bio-One) and cryopreserved in FCS (HyClone) containing 10% DMSO (Sigma-Aldrich) or were used immediately.

Reagents

Recombinant soluble human LAG-3Ig fusion protein (IMP321), a 200-kDa dimer, was produced in Chinese hamster ovary cells transduced with a plasmid encoding for the extracellular domain of human LAG-3 fused to the human IgG1 Fc. The purified bulk preclinical batch has a concentration of 1.9 mg/ml IMP321 and 0.75 EU/mg of endotoxin, 0.42 ng/ml DNA, and 6 ng/mIgG1 and simultaneously stained with a 0.5% azide for 30 min at 4°C to block host cell proteins. Human IgG1 (hIgG1) was obtained from Chemicon International. IMP321 and hlgG1 were conjugated to Alexa Fluor 488 using the FluoReporter protein labeling kit from Molecular Probes. The series of TLR agonists was purchased from Apotech. The anti-LAG-3 mAb (clone 17B4, IgG1) directed to the extra loop of the Ig-like domain 1 of LAG-3 (18) has been characterized previously (19, 20). This mAb was FITC labeled using FluoReporter FITC protein labeling kit from Molecular Probes. A second anti-LAG-3 mAb (clone A9H12, IgG2a) was obtained by immunization of mice with CHO cells transduced with LAG-3 and boosted with sLAG-3. Isotype-matched control mAbs (mIgG2a and mIgG1) were purchased from Southern Biotechnology Associates. The M-DC8 mAb (IgM) has been characterized previously (21, 22). FITC-conjugated anti-IFN-γ (25723.11), TNF-α (6401.1111), granzyme A (CB9) and granzyme B (GB11), PE-conjugated CD1a (SK9), CD11b (D12), CD11c (B-ly6), CD13 (Leu-M7), CD14 (Mpd9), CD16 (Leu 11c), CD33 (P67.6), CD45RA (HI100), CD56 (MY31), CD123 (9F5), anti-HLA-DR (L243), TCRγTD2 (11F2), MCP-1 (5D3-F7), CCL4/MIP-1β (D21-1351), IL-2 (5344.11), and IFN-γ (25723.11), PerCP-Cy5.5-conjugated CD3 (SK7) and CD19 (J25C1), PerCP-conjugated CD14 (Mdp9), PE-Cy7-conjugated CD4 (SK3), CD16 (B73.1), CD45RA (L48), and CD56 (B159), allopoycocyanin-conjugated anti-TNF-α (6401.1111), CD11a (HI111), CD11c (S-HCL-3), CD14 (Mpd9), and CD27 (L128), Alexa 647-conjugated anti-CCR7 (3D12) and allopoycocyanin-Cy7-conjugated CD8 (SK1) and anti-HLA-DR (L243) mAbs, brefeldin A, CytoFix/CytoPerm, and Perm/Wash buffers were purchased from BD Biosciences. PE-conjugated CD4 (13B8.2), CD80 (MAB104), CD83 (HI15a), and CD86 (HA5.2/87) were obtained from Coulter Immunotech and PE-conjugated anti-HLA-DR/CD11c, CD14/CD16, CD11c/CD16, HLA-DR/CD16, and CD3/CD14 antibodies were used to block specific binding of immune markers.

Binding assay of IMP321

PMBCs were incubated with M-DC8/anti-IgM-PE, then incubated with Alexa 488-hlgG1 (dashed line) or Alexa 488-IMP321 (filled histogram) (3 μg/ml), stained with CD14-PerCP, CD11c-allophycocyanin, CD16-PE-Cy7, and HLA-DR-allophycocyanin-Cy7 mAbs and analyzed on a six-color cytometer. After exclusion of doublets, debris, and dead cells based on forward and side scatter parameters, IMP321 was shown in gray and IMP321 (black) cells were gated and analyzed separately (A, left). Dot plots showing the expression of HLA-DR/CD11c, CD14/CD16, CD11c/CD16, HLA-DR/CD16, and IMP321/M-DC8 of IMP321 cells (gray) and IMP321 cells (black) are presented in the right panels. Data are representative of at least four donors.

Inhibition of Alexa 488-IMP321 binding to PBMCs was done by preincubation of Alexa 488-IMP321 with an anti-LAG-3 (black line) vs a control Ab (filled histogram). Then the mixture was used to stain the cells as described above (B).
Cytometric bead array (CBA) assay

PBMCs were incubated in complete RPMI 1640 containing 10% FCS with or without IMP321 (1 μg/ml or indicated concentration), hlgG1 (1 μg/ml), anti-MHC class II mAb (1 μg/ml, clone I3; Coulter), or TLR agonists (concentrations are indicated as optimal by the provider): TLR1-2 (Pam3Cys-Ser-(Lys)4, 0.1 μg/ml), TLR3 (polyinosinic-polycytidylic acid, 100 μg/ml), TLR4 (LPS, 1 μg/ml), TLR5 (flagellin, 0.1 μg/ml), TLR6-2 (macrophage-activating lipopeptide-2, 0.1 μg/ml), TLR7-8 (polyuridylic acid, 10 μg/ml), or TLR9 (CpG oligodeoxynucleotide 2395, 10 μg/ml) for 24 or 48 h as indicated. IFN-γ, TNF-α, IL-1β, IL-6, IL-10, CCL2, CCL4, and CCL5 were quantified in culture supernatants by specific CBA Flex sets according to manufacturer's instructions (BD Biosciences). Data were acquired with FACSCanto cytometer and cytokines/chemokines concentrations were calculated using FCAP software (BD Biosciences). Results are reported as a quantity of analytes produced by 10⁶ cells.

PBMC stimulation and intracellular staining

PBMCs were stimulated with IMP321 (1 μg/ml or indicated concentration), hlgG1 (1 μg/ml), anti-MHC II mAb (1 μg/ml, clone L243, or clone I3; BD Biosciences) or TLR agonists (see previous section) in complete RPMI 1640 10% FCS for 18 h in the presence of brefeldin A during the last 6 h for or 4 h in the presence of brefeldin A (to evaluate myeloid cells activation) in a humidified incubator at 37°C. In neutralization assays, indicated concentrations of IMP321 were incubated with anti-LAG-3 mAb (17B4, 10 μg/ml) or control mlgG1 (10 μg/ml) for 30 min at room temperature before adding the cells. After stimulation, cells were washed and labeled with fluorochrome-conjugated specific Abs for CD4, CD8, CD11a, CD11c, CD14, CD16, CD27, CD28, CD45RA, HLA-DR, and/or CCR7 as indicated in the figure legends. Cells were fixed and permeabilized using CytoFix/Cytoperm, washed with Perm/Wash buffer, and then stained for cytokines/chemokines, and CD3 when required. After extensive washing with Perm/Wash buffer, cells were analyzed using a six-color FACSCanto flow cytometer to determine the percentage of cells expressing cytokines/chemokines and to phenotype the corresponding activated cells. The results following FACS analysis are defined as the differences between the percentage of cells expressing at least one cytokine upon IMP321 stimulation and the percentage of cytokine expression in basal condition.

**FIGURE 2.** IMP321 induces PBMCs to produce cytokines/chemokines. Quantitative measurement of IFN-γ, TNF-α, IL-1β, IL-6, IL-10, CCL2, CCL4, and CCL5 in 48-h supernatants of unstimulated (medium) and IMP321-stimulated (1 μg/ml) PBMCs from 24 donors (9 for CCL2) were determined by CBA assays. Data represent the concentrations of cytokine/chemokine for each donor (circles) and the medians obtained for all donors (bars), expressed in picograms per 10⁶ cells on a logarithmic scale (A). Eight PBMC samples were stimulated with the indicated concentrations of IMP321 for 24 h and the concentration of CCL4 in the supernatants was determined by CBA. Data represent the mean ± SD of CCL4 concentrations determined for all PBMC samples, expressed in picograms per 10⁶ cells on a logarithmic scale and plotted as a function of IMP321 concentration (B).
**Isolation of CD8\(^+\) T cell populations from PBMCs**

PBMCs were thawed and stained with PE-conjugated CD4, CD14, CD16, CD56, anti-TCR\(\gamma\)/anti-TCR\(\delta\), HLA-DR and HLA-DR/DQ/DP, CD3-PerCP-Cy5.5 and CD8-allophycocyanin-Cy7, washed, and CD3\(^-\)/CD8\(^-\) cells were sorted after exclusion of debris, doublets, and PE-positive cells using a FACSaria cell sorter (BD Biosciences). Resulting preparations were consistently 96% CD3\(^+\)/CD8\(^+\) and the remaining events consisted of debris. Whole PBMCs and CD3\(^-\)/CD8\(^-\) sorted cells were then incubated with or without IMP321 for 18 h with brefeldin A for the last 16 h, stained for CD3, CD4, CD8, IFN-\(\gamma\), and TNF-\(\alpha\) and analyzed by cytometry as described above.

**Proliferation assay**

Fresh PBMCs were washed in PBS, resuspended at 2 × 10\(^6\)/ml in PBS, stained with CFSE (50 nM) for 10 min at 37°C in the dark and washed in RPMI 1640 containing 10% FCS (Invitrogen Life Technologies). Cells were incubated with or without IMP321 (10 \(\mu\)g/ml) at 37°C. After 4 days, T cell subpopulations were stained with CD4-PE-Cy7 and CD8-allophycocyanin-Cy7 Abs and analyzed by flow cytometry. The percentage of CD4\(^+\) and CD8\(^+\) CFSE\(^{\text{low}}\) cells corresponding to dividing cells was determined using the ModFit software (Verity Software House). Weighted scores are calculated by multiplying the total percentage of dividing CFSE\(^{\text{low}}\) cells by the number of cell divisions.

**Results**

**Binding of IMP321 to PBMCs**

Alexa 488-labeled IMP321 bound to a minority (<10%) of MHC class II\(^+\) cells, representing 0.5–5.6% of total PBMCs in seven experiments (Fig. 1A, upper panel). IMP321\(^-\) and IMP321\(^+\) cells were then phenotyped separately. In the IMP321\(^-\)/HLA-DR\(^+\)/CD11c\(^-\) population, the percentages of CD16\(^+\)/CD14\(^-\) or CD16\(^+\)/CD14\(^+\) were variable from one subject to another but these remained the main subsets (Fig. 1A, right panels). This IMP321\(^-\)/HLA-DR\(^+\)/CD11c\(^+\)/CD16\(^-\)/CD14\(^+\) subset also expressed CD4, CD13, CD33, CD45RA, and CD86 but not CD1a, CD3, CD19, CD80, CD83, or CD123 (data not shown). Thus, the IMP321\(^+\) cell subset includes cells phenotypically defined as myeloid CD16\(^+\)/DCs (23). Of note, all myeloid CD11c\(^+\)/CD16\(^-\) DCs in PBMCs as well as almost all M-DC8\(^+\) cells, the major DC subpopulation in blood (21, 22), were IMP321\(^-\). In addition, a small percentage of IMP321\(^-\) cells were HLA-DR\(^+\)/CD11c\(^-\)/CD16\(^+\)/CD14\(^+\) monocytes (Fig. 1, right panel) and accordingly most monocytes were IMP321\(^+\) cells even though they express MHC class II molecules. The same happens for B lymphocytes with only a small percentage (<5%) of CD19\(^+\) cells being IMP321\(^+\) (data not shown). Preincubation of IMP321 with a LAG-3 Ab inhibited its binding to PBMCs (Fig. 1B) showing that the LAG-3 domain and not theFc portion was responsible for its binding to APCs. Also, the three CD16 dot-plot histograms in Fig. 1 clearly show that cells lacking this FcR do bind IMP321.

**IMP321 induces PBMCs to produce cytokines/chemokines, but not IL-10**

The individual quantitative analysis (CBA assay) of a series of 48-h PBMC supernatants showed that IMP321 at 1 \(\mu\)g/ml consistently induced a 1–3 orders-of-magnitude increase in the concentrations of IFN-\(\gamma\), TNF-\(\alpha\), IL-1\(\beta\), IL-6, CCL4, CCL2, and CCL5 (Fig. 2A). Of note, IL-10 was not induced and in some donors even decreased compared with the unstimulated condition (Fig. 2A). In dose-response experiments, the increase in CCL4 at 24 h was detected with IMP321 concentrations as low as 10 ng/ml (Fig. 2B) whereas stimulation of PBMC with 1 \(\mu\)g/ml hlgG1 had no effect (\(n = 8\), data not shown). In the same conditions, cross-linking MHC class II molecules with a specific mAb (clone I3) at 1 \(\mu\)g/ml induced only a slight increase (<10% of the IMP321 effect) of IFN-\(\gamma\), TNF-\(\alpha\), IL-1\(\beta\), CCL2, and CCL5 (\(n = 4\), data not shown). Of note, IL-10 was not induced and in some donors even decreased compared with the unstimulated condition (Fig. 2A). In dose-response experiments, the increase in CCL4 at 24 h was detected with IMP321 concentrations as low as 10 ng/ml (Fig. 2B) whereas stimulation of PBMC with 1 \(\mu\)g/ml hlgG1 had no effect (\(n = 8\), data not shown). In the same conditions, cross-linking MHC class II molecules with a specific mAb (clone I3) at 1 \(\mu\)g/ml induced only a slight increase (<10% of the IMP321 effect) of IFN-\(\gamma\), TNF-\(\alpha\), IL-1\(\beta\), CCL2, and CCL5 (\(n = 4\), data not shown).

**IMP321 induces myeloid cells to produce cytokines/chemokines**

The effect of IMP321 on cytokine/chemokine production in different cell subsets was assessed by intracellular staining followed by flow cytometry analysis. PBMCs were stimulated with IMP321 for 4 h, stained with HLA-DR and CD11c mAb, and permeabilized to detect the intracellular expression of TNF-\(\alpha\) and CCL4. IMP321, but not the control hlgG1, induced the expression of CCL4 and/or TNF-\(\alpha\) in ~5% of PBMCs (Fig. 3, upper panel). Cells expressing CCL4 and TNF-\(\alpha\) are shown in black, cells expressing one of these cytokines are shown in dark gray, and cells expressing none of these cytokines are shown in gray. Lower panel. The expression of HLA-DR/CD11c on the CCL4\(^+\)/TNF-\(\alpha\) cells (black), the single cytokine\(^+\) cells (dark gray) and the cytokines\(^+\) cells (gray). Results are representative of six donors.

**IMP321 induces CD8\(^+\) T cells and NK cells to produce cytokines/chemokines**

After PBMC stimulation (60 donors) with IMP321 for 18 h, cells were stained with CD3, CD4, CD8 mAb and with IFN-\(\gamma\), TNF-\(\alpha\), and IL-2 mAb after permeabilization of the cell membrane. The percentages of CD3\(^-\)/CD4\(^+\), CD3\(^+\)/CD8\(^+\) T cells, and CD3\(^-\)/CD8\(^+\) NK cells expressing one, two, or three of these cytokines were determined. The measurement of cytokine\(^+\) cells using this procedure has been validated previously and gives an overall coefficient of variation <20% after Ag-specific stimulation (24). A slight increase in cytokine-expressing CD4\(^+\) T cells was detected in 44 (of 60) PBMCs stimulated with IMP321 compared with medium alone. Of these, 14 displayed a significant increase (data not shown). In contrast, a much higher percentage of cytokine-expressing CD8\(^+\) T cells was observed (Fig. 4A, upper panel).
Indeed, IMP321 induced a significant percentage of IFN-γ or IFN-γ, TNF-α activated CD8 T cells in most donors (46 of 60). We have compared this to the response of CD8 T cells to a pool of CMV peptides in 24 of these 60 donors and found that the range of values shown in Fig. 4A is quite similar to the one observed in response to CMV stimulation (data not shown). The effect of IMP321 was also determined on CD3,CD8 and CD3,CD8 cells for one donor is shown as an example in Fig. 4B (upper panel).

**FIGURE 4.** Induction of activated CD8 T and NK cells by IMP321 in PBMCs from 60 healthy donors and 21 metastatic cancer patients. PBMCs incubated with IMP321 (1 μg/ml) for 18 h were stained with CD4-PE-Cy7, CD8-allophycocyanin-Cy7, fixed, permeabilized and stained with CD3-PerCP-Cy5.5, anti-IFN-γ-FITC, IL-2-PE, and TNF-α-allophycocyanin mAbs. The percentage of CD3,CD8 T lymphocytes and CD3,CD8 NK cells expressing IFN-γ, TNF-α, and/or IL-2 was determined by flow cytometry. Percentage of cells expressing cytokines in unstimulated conditions was subtracted from IMP321-stimulated percentage. Because no significant IL-2 production was induced in CD3,CD8 or CD3,CD8, these data are not presented. The percentages of IFN-γ, TNF-α, IFN-γ, TNF-α, and IFN-γ, TNF-α in CD3,CD8 and CD3,CD8 subpopulations are presented for every subject (A). An example of IFN-γ and TNF-α expression in CD3,CD8 (B, upper left) and CD3,CD8 cells (B, upper right) in one donor and the mean ± SEM of the percentage of cells expressing IFN-γ and/or TNF-α for the 60 healthy donors shown (B). In dot plots, the percentages of cells in IMP321-stimulated and unstimulated conditions are indicated in each quadrant in bold and in parentheses, respectively. PBMCs from 21 metastatic cancer patients were stimulated or not with IMP321 (1 μg/ml) and then were stained and analyzed as described in A. Data represent the percentage of CD3,CD8 (C, left) and CD3,CD8 cells (C, right) expressing at least one of the cytokines (IFN-γ, IL-2, and TNF-α) for healthy donors ≥40 years old and the 21 patients as indicated. Bars represent the mean in each group. *, A significant difference between the two groups (Student’s t test, p < 0.05).
cells population (CD3<sup>+</sup>) were stimulated with or without IMP321 (10 ng/ml) and stained with CD3-PerCP-Cy5.5, anti-IFN-γ, CD56-PE-Cy7, CD8-allophycocyanin-Cy7, fixed, permeabilized, and then stained with CD3-PerCP-Cy5.5, anti-IFN-γ-FITC, and TNF-α-allophycocyanin mAbs to show the expression of these cytokines in the whole NK cells population (CD3<sup>+</sup>CD56<sup>+</sup>), the CD8<sup>+</sup> NK cells subset (CD3<sup>−</sup>CD8<sup>+</sup>), the CD8<sup>+</sup> T cells and NK cells to produce cytokines/chemokines. PBMCs (from one representative subject of six tested) were stimulated with or without IMP321 (10 ng/ml) and stained with CD4-PE-Cy7, CD8-allophycocyanin-Cy7, fixed, permeabilized, and then stained with CD3-PerCP-Cy5.5, anti-IFN-γ-FITC, and TNF-α-allophycocyanin mAbs to show the expression of these cytokines in the whole CD8<sup>+</sup> T cells (Fig. 5, A). IMP321 also induces the production of CCL4 in a large percentage of CD8<sup>+</sup> T cells (26%) in the three donors analyzed (Fig. 5, A, left panel, C) while the percentages of CD8<sup>+</sup> NK cells were significantly lower in cancer patients compared with healthy donors (Fig. 4C, right panel).

Similar frequencies were observed for CD3<sup>+</sup>CD8<sup>+</sup>TCR<sup>+</sup>/CD3<sup>+</sup>CD8<sup>+</sup>TCR<sup>−</sup> T cells (data not shown) or for the whole NK population (CD3<sup>−</sup>CD56<sup>+</sup> cells; Fig. 5A). IMP321 also induces the production of CCL4 in a large percentage of CD8<sup>+</sup> T cells (3.7% vs 0.4% for CD3<sup>+</sup> CD8<sup>+</sup> cells, respectively). The Tc1 activation of CD8<sup>+</sup> T cells by IMP321 was detectable at concentrations as low as 30 ng/ml and was inhibited by the prior treatment with IMP321-stimulated PBMCs were stained with CD4-PE-Cy7, CD8-allophycocyanin-Cy7, fixed, permeabilized, and then stained with CD3-PerCP-Cy5.5, anti-IFN-γ, and TNF-α mAbs (L243 or I3, n = 2) had any effect on the activation of CD8<sup>+</sup> T cells by IMP321. PBMCs were first labeled with CFSE, incubated with IMP321, and analyzed on day 4 for the number of cell divisions. IMP321 induced more CD8<sup>+</sup> T cells to divide (10 and 8% compared with 4 and 3%, respectively, for the two donors shown in Fig. 6A). The Tc1 activation of CD8<sup>+</sup> T cells by IMP321 was detected at concentrations as low as 30 ng/ml and was inhibited by the prior addition to the PBMCs of a blocking LAG-3 mAb (Fig. 6B). Pure sorted populations of CD3<sup>−</sup>CD8<sup>+</sup> cells could not be induced by IMP321 to produce IFN-γ or TNF-α (Fig. 6C), underlining the role of MHC class II<sup>+</sup> APC activation by IMP321 in Tc1 activation.

Effect of IMP321 on T cell proliferation
In addition to these early cell activation events, we also studied the later proliferative effect of IMP321. PBMCs were first labeled with CFSE, incubated with IMP321, and analyzed on day 4 for the number of cell divisions. IMP321 induced more CD8<sup>+</sup> T cells to divide (10 and 8% compared with 4 and 3%, respectively, for the two donors shown in Fig. 6A). In addition to this, the proliferating cells divide more (up to generation 5) in the presence of IMP321. Weighted scores calculated by multiplying the total percent divisions of CFSE<sub>low</sub> by the number of cell divisions were 5 and 3% in unstimulated condition compared with 18 and 12% for IMP321-stimulated CD8<sup>+</sup> cells. The same proliferative effect of IMP321 was also observed on CD4<sup>+</sup> T cells but to a lesser extent (data not shown).

The IMP321-stimulated cells are Ag-experienced effector or effector memory CD8<sup>+</sup> T cells
To further characterize the specific population of IMP321-induced IFN-γ<sup>+</sup>TNF-α<sup>+</sup>CD8<sup>+</sup> T cells, PBMCs were analyzed at 18 h to discriminate naive, effector, effector memory, and central memory (CM) T cell subsets. Naive T cells were not stimulated by IMP321, CD3<sup>−</sup>CD8<sup>+</sup>) vs the CD8<sup>+</sup> NK cells subset (CD3<sup>−</sup>CD56<sup>+</sup>CD8<sup>+</sup>) (A). IMP321-stimulated PBMCs were stained with CD4-PE-Cy7, CD8-allophycocyanin-Cy7, fixed, permeabilized, and then stained with CD3-PerCP-Cy5.5, anti-IFN-γ-FITC, CCL4-PE, and TNF-α-allophycocyanin mAbs (B and C). Dot plot histograms showing the expression of IFN-γ, TNF-α, and CCL4 in CD1<sup>+</sup> CD8<sup>+</sup> cells (B) and CD3<sup>+</sup> CD8<sup>+</sup> cells (C) in a representative subject (of three) are presented. In dot plot histograms, the percentages of cells in IMP321-stimulated and unstimulated conditions are indicated in each quadrant in bold and in parentheses, respectively.
IMP321 induces a full Tc1 activation but no IL-10 production, in contrast to TLR agonists

PBMCs from four donors were stimulated with IMP321 or with the following TLR agonists at their optimal concentrations: TLR1-2 (lipopeptide), TLR3 (dsRNA), TLR4 (LPS), TLR5 (flagellin), TLR6-2 (macrophage-activating lipopeptide-2), TLR7-8 (ssRNA), or TLR9 (CpG). At these concentrations, all the TLR agonists were found to be at least as potent as 1 μg/ml IMP321 at inducing high IFN-γ, TNF-α, IL-1β, IL-6, CCL4, CCL5, and CCL2 concentrations in 48-h supernatants (data not shown). The immunosuppressive cytokine, IL-10, was only induced by the TLR ligands (Fig. 8A) but not by IMP321 (Fig. 2 and four additional donors in Fig. 8A). In fact, IMP321 even decreased the level of IL-10 secretion in some donors compared with the unstimulated condition (Figs. 2 and 8A).

In line with this IL-10 induction, the TLR agonists were unable to induce a full Tc1 activation with no IFN-γ-CD8+ T cells being induced at 18 h, compared with IMP321 (Fig. 8B). Only an elevated inflammatory background type of response (i.e., expression of TNF-α by some T cells) was induced by TLR agonists (Fig. 8B). Similarly, IMP321, but not the TLR agonists, induces IFN-γ-TNF-α+ cells in the CD3-CD56- NK subset (10 PBMC samples tested, data not shown). Overall, these data indicate that the lack of IL-10 induction by IMP321 correlates with its ability to induce IFN-γ-TNF-α+ Tc1-type CD8 and NK effector cells, in contrast to TLR agonists.
**Discussion**

IMP321 is a clinical grade LAG-3Ig recombinant protein which binds to a small subset of blood myeloid cells, including all myeloid DC and a minor percentage of MHC class II+ monocytes. Less than 10% of MHC class II+ PBMCs are labeled by LAG-3Ig while all transformed B cells in culture (e.g., DAUDI, RAJI) are strongly stained (25). The partition of MHC class II molecules in cholesterol-enriched microdomains is apparently critical to the binding of LAG-3Ig on immature human DC (2, 4), leading in few minutes to the phosphorylation of phospholipase C and p72^syk^ as well as the activation of the PI3K/Akt pathway involved in inducing the maturation process of LAG-3-stimulated DCs in vitro.
These observations were made on healthy subject PBMCs and it posed of 6-sulfo LacNAc-expressing DC (21, 22), and monocytes. The percentage capacity (27, 28). Indeed, Ag-experienced effector or EM T cells, NK cells, myeloid DCs (23) mainly composed of 6-sulfo LacNAc-expressing DC (21, 22), which are known to be directly cytotoxic for tumor cells when activated by CCL5, all agonists of CCR5, polarize the immune response by influencing indirectly through APCs to drive the immune response to mature DC and the same cells matured in vitro has been reported (5). It is thus possible that the partitioning of MHC class II is different between various types of APC subsets in vivo, explaining the selective binding of IMP321 to some primary, unmanipulated APCs and not to others. The major subset of PBMCs strongly labeled by IMP321 was HLA-DR+ CD11c+ CD16+ CD14− int, including the major population of circulating DC (~2% in blood), the M-DC8+ 6-sulfo LacNAc-expressing DC (21, 22) which are known to be directly cytotoxic for tumor cells when activated by IFN-γ (26).

A short exposure to IMP321 (2–4 h) was sufficient to induce the expression of chemokines/ cytokines in unmanipulated APCs. In addition to the crucial involvement of IFN-γ, T and NK cell activation and differentiation, many chemokines influence the outcome of the immune response. For instance, CCL3, CCL4, and CCL5, all agonists of CCR5, polarize the immune response by acting indirectly through APCs to drive the immune response toward a Th1/1c1 pattern, in addition to their T lymphocyte attraction capacity (27, 28). Indeed, Ag-experienced effector or EM CD8+ T cells that were fully differentiated, as shown by the lack of CD27 and CD28 expression (29), were driven by IMP321 into full Tc1 activation after 18 h (a result that could not be obtained with TLR agonists) representing, as a mean, 1% of CD8-circulating T cells. The same applied for NK cells with, as a mean, 3.7% of NK being recruited into the pool of functionally active cytotoxic-type cells. Regarding the mechanism of action, we were unable to induce Tc1 activation of CD8 T and NK cells using supernatants of PBMCs previously incubated for 24 h with IMP321 and then depleted of IMP321 using protein A beads (data not shown). It is thus possible that this IMP321 effect requires cell-cell interaction. Overall, the percentage of PBMCs specifically expressing such type 1 cytokines upon stimulation by 1 μg/ml IMP321 represented 2.1 ± 0.2% (mean ± SEM). These activated PBMCs included a large array of potentially cytotoxic cells, namely CD8+ T cells, NK cells, myeloid DCs (23) mainly composed of 6-sulfo LacNAc-expressing DC (21, 22), and monocytes. These observations were made on healthy subject PBMCs and it remains to be seen whether this will also apply to cancer patient PBMCs. In the present study, we have shown in a small series of 21 untreated metastatic cancer patients that the level of induction of activated NK cells (but not CD8 T cells) by IMP321 was reduced compared with healthy subjects (Fig. 4C).

In human blood, most LAG-3+ cells are natural CD4+CD25high FoxP3+ regulatory T cells (Treg) (30, 31) and blocking LAG-3 signaling into these cells is known to increase T cell proliferation, Th1/1c1 differentiation, and antitumor cytotoxicity (30, 32–34). Therefore, the question arises as to whether the effect of IMP321 on recruiting and re-energizing different effector cells through APC activation may be being reinforced through blocking of LAG-3 signaling into Tregs leading to Treg silencing. The mechanism for this could be either direct competition for the MHC class II subset receptors or, more drastically, the rapid internalization of those receptors, which is triggered by the LAG-3 binding. We believe that the latter is likely to be the case at the 1 μg/ml IMP321 concentration we used in functional assays because after their incubation at 37°C for only 15 min, IMP321-labeled PBMCs are no longer detected (data not shown) which could be due to this rapid internalization of their very restricted subset of MHC class II receptors. In fact, this direct neutralization of Treg activity is supported by the lack of IL-10 induction by IMP321 in contrast to TLR ligands, explaining in part why fully differentiated CD8 effector T cells could be re-energized to secrete IFN-γ by IMP321 but not by TLR agonists. Recent studies have underlined the role of IL-10 in suppressing the establishment of robust cellular immune responses able to clear viral infection in mice (35, 36). In humans, IL-10 has been shown to be secreted in considerable amounts by virus-specific CD8 Tr1 cells infiltrating the liver of patients with chronic hepatitis C virus infection, a disease where T cell responses are typically down-modulated (37).

What are the implications of these findings? They support the view that non-TLR ligands such as IMP321 may be used to recruit a large range of effector cells in both innate and acquired immune responses to tumor cells. The clinical development of soluble CD40L has been hampered by an increased risk of
thrombosis due to platelet activation (38) but CD40 agonist therapeu-
tic Abs are now being tested in cancer patients. IMP321 has been
shown to be safe and well-tolerated in several phase I/II trials
(www.clinicaltrials.gov) (24, 39) and immunomonitoring will re-
veal whether some of the effector cell subsets described in the
present study are indeed activated in vivo.

Acknowledgment

We thank Dr. Jean-Pierre Aubry from BD Biosciences for cell sorting and
advice.

Disclosures

FT has some equity interests in Immutep S.A. CB, CG, MM and FT are
current employees of Immutep S.A.

References

Lympocyte activation gene-3, a MHC class II ligand expressed on activated T
cells, stimulates TNF-α and IL-12 production by monocytes and dendritic cells.
168: 3874–3880.
(CD223) leads to a distinct pattern of chemokine receptor expression by human
in human dendritic cells induced by a natural ligand, the LAG-3 protein (CD223).
5. Seidm, N., C. Rouard, C. Bocaccio, J. P. Abastado, D. Charron, and
N. Mooney. 2003. Composition of MHC class II-enriched lipid microdomains
is modified during maturation of primary dendritic cells. J. Leukocyte Biol.
74: 40–48.
I presentation of exogenous antigen by dendritic cells is controlled by CD4+
T cells engaging class II molecules in cholesterol-rich domains. J. Immunol.
168: 1172–1180.
MHC class II-peakptides in dendritic cell lipid microdomains initiate the
adjuvant elicits greater humoral and cellular immune responses to both particulate
3867–3875.
E. Di Carlo, P. M. Musiani, G. Vignali, and G. Foroni. 2003. LAG-3 enables DNA
vaccination to persistently prevent mammary carcinogenesis in HER-2/neu trans-
M. Giovarelli, G. Forni, P. Musiani, and F. Triebel. 2005. Immunological mech-
isms elicited at the tumor site by lymphocyte activation gene-3 (LAG-3) versus
IL-12: sharing a common Th1 anti-tumor immune pathway. J. Pathol. 205:
82–91.
12. Casati, C., C. Camisaschi, F. Rini, F. Arienti, L. Rivoltini, F. Triebel,
G. Parmiani, and C. Castelli. 2006. Soluble human LAG-3 molecule amplifies the
in vitro generation of type 1 tumor-specific immunity. Cancer Res. 66:
4450–4460.
immunotherapy for therapeutic vaccines: preclinical evaluation of IMP321.
Vaccine 24: 5426–5433.
lymphocyte activation gene 3-encoded protein: a new ligand for human leucocyte
expression and tissue distribution of the human LAG-3-encoded protein, a MHC
B. Maigret, M. Dreano, and F. Triebel. 1997. Characterization of the major his-
ocompatibility complex class II binding site on LAG-3 protein. Proc. Natl. Acad.
1998. A novel dendritic cell population in human blood: one-step immunomag-
netic isolation by a specific mAb (MCDC8) and in vitro culturing of cytotpic T
6-sulfo LacNAc-expressing dendritic cells are principal producers of early inter-
leukin-12 and are controlled by trycrocystes. Immunity 24: 767–777.
19. MacDonald, K. P., D. J. Munster, G. J. Randolph, C. F. Neurohr, and D. N.
100: 4512–4520.
(sLAG-3), an immunopotentiator for T cell responses against a HBsAg antigen in
healthy adults: a single blind randomised controlled phase 1 study. J. Immunol
Based Ther. Vaccines 5: 5.
major histocompatibility complex class II interaction analyzed with CD4- and
22. Schakel, K., S. Zhao, Y. Deuse, K. Schler, R. Wehner, H. Wohner, K. Holig,
native blood dendritic cells: direct tumor cell killing and activation of NK cell-
23. Loetscher, P., M. Ugliccioni, L. Bordoli, M. Taggazioni, B. Moser, C. Chizzolini,
and J. M. Dayer. 1998. CCR5 is characteristic of Th1 lymphotoes. Nature 391:
344–345.
24. Dorner, B. G., A. Scheffold, M. S. Rolph, M. B. Huser, S. H. Kaufmann,
A. Radbruch, I. E. Flesch, and R. A. Kroc. 2002. MIP-1α, MIP-1β, RANTES,
and NF-κB activity linked to CD40 ligand expression and tissue distribution of the
human LAG-3-encoded protein, a MHC class II ligand. J. Exp. Med. 195:
2141–2147.
25. Loetscher, P., M. Ugliccioni, L. Bordoli, M. Baggiolini, B. Moser, C. Chizzolini,
and J. M. Dayer. 1998. CCR5 is characteristic of Th1 lymphocytes. Nature 391:
344–345.
6-sulfo LacNAc-expressing dendritic cells are principal producers of early inter-
leukin-12 and are controlled by trycrocystes. Immunity 24: 767–777.
27. MacDonaIl, C. P. J. Munster, G. J. Randolph, C. F. Neurohr, and D. N.
100: 4512–4520.