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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 Schäkel,† and Frédéric Triebel*†

The principal antitumor immune response is mediated through the activation of type 1 cytotoxic (Tc1) CD8 T cells, NK cells, and monocytes/macrophages. In this study, we investigated the potency of a clinical-grade soluble form of lymphocyte activation gene-3 protein (IMP321), a physiological high-affinity MHC class II binder, at inducing in PBMCs an appropriate cytotoxic-type response in short-term ex vivo assays. We found that IMP321 binds to a minority (<10%) of MHC class II+ cells in PBMCs, including all myeloid dendritic cells, and a small fraction of monocytes. Four hours after addition of IMP321 to PBMCs, these myeloid cells produce TNF-α and CCL4 as determined by intracellular staining. At 18 h, 1% of CD8+ T cells and 3.7% NK cells produce Tc1 cytokines such as IFN-γ and/or TNF-α (mean values from 60 blood donors). Similar induction was observed in metastatic cancer patient PBMCs, but the values were lower for the NK cell subset. Early APC activation by IMP321 is needed for this Tc1-type activation because pure sorted CD8+ T cells could not be activated by IMP321. Only Ag-experienced, fully differentiated granzyne+ CD8 T cells (effector and effector memory but not naive or central memory T cells) are induced by IMP321 to full Tc1 activation. In contrast to IMP321, TLR1-9 agonists induce IL-10 and are therefore unable to induce this Tc1 IFN-γ* response. Thus, IMP321 has many properties that confirm its potential to be a new class of immunopotentiator in cancer patients. The Journal of Immunology, 2007, 179: 4202–4211.

An 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+ T 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

Abbreviations used in this paper: Tc1, type 1 cytotoxic T cell; DC, dendritic cell; LAG-3, lymphocyte activation gene-3; sLAG-3, serum protein LAG-3; hIgG1, human IgG1; CBA, cytometric bead array; CM, central memory; EM, effector memory; Treg, regulatory T cell.
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 AB blood pools 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 transfected 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/ml host cell proteins. Human IgG1 (hIgG1) was obtained from Chemicon International. IMP321 and hIgG1 were conjugated to Alexa Fluor 488 using the FluorReporter 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 FluorReporter 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 transfected with LAG-3 and boosted with sLAG-3Ig. 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), CD1b (D12), CD11c (B-ly6), CD13 (Leu-M7), CD14 (Mdp9), CD16 (Leu 11c), CD33 (P67.6), CD45RA (HI100), CD56 (MY31), CD123 (9F5), anti-HLA-DR (L243), TCRyTcRδ (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), allophycocyanin-conjugated anti-TNF-α (6401.1111), CD11a (HI111), CD11c (S-HCL-3), CD14 (Mdp9), and CD27 (L128), Alexa 647-conjugated anti-CCR7 (3D12) and allophycocyanin-Cy7-conjugated CD8 (SK1) and anti-HLA-DR (L243) mAbs, brefeldin A, CytoFix/CytoPerm, and Perm/Wash buffers were purchased from BD Biosciences. PE-Cy7-conjugated CD4 (13B8.2), CD80 (MAB104), CD83 (HIB15a), and CD86 (HA5.2B7) were obtained from Coulter Immunotech and PE-conjugated anti-HLA-DR/DQ/DP (WR18) mAb was obtained from Abcam.

#### Binding assay of IMP321

PMBCs were incubated with MDC-8 mAb, washed, and labeled with anti-IgM-PE (Immunotech). Then cells were incubated in PBS containing 10% human serum (Bio Media), 0.1% azide for 30 min at 4°C to block unspecific binding sites, and then with Alexa 488-conjugated IMP321 or hlgG1 at a final concentration of 3 μg/ml in PBS, 0.5% BSA, 0.1% azide containing 30 μg/ml unlabelled hlgG1 and simultaneously 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− (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 3 h. 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+ cells in unstimulated conditions. The confidence interval depended on the numbers of relevant events (CD3⁺ CD8⁺ or CD3⁺ CD8⁺ events) collected in each sample, the background expression of cytokines in unstimulated PBMC and the difference between the two conditions. This difference was significant with a power of 90% (p < 0.05) if the number of CD3⁺ CD8⁺ or CD3⁺ CD8⁺ cells collected by the cytometer in a given sample was larger than calculated events using the formula:

\[2 \times \left((\text{Stim}/100 + \text{Unstim}/100)\right) \times (1 - (\text{Stim}/100 + \text{Unstim}/100)/2) \times 8.6/(\text{Stim}/100 - \text{Unstim}/100)^2\]

where Stim is the percentage of CD3⁺ CD8⁺ or CD3⁺ CD8⁺ cells expressing at least one cytokine upon stimulation with IMP321 and Unstim is the percentage of CD3⁺ CD8⁺ or CD3⁺ CD8⁺ cells expressing at least one cytokine in basal condition.
Isolation of CD8+ T cell populations from PBMCs
PBMCs were thawed and stained with PE-conjugated CD4, CD14, CD16, CD56, anti-TCR/anti-TCRβ, 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-γ, and TNF-α 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 μg/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+ CFSElow 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 CFSElow 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). The main 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+ cells (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 the Fc 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 μg/ml consistently induced a 1–3 orders-of-magnitude increase in the concentrations of IFN-γ, TNF-α, IL-1β, 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 μg/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 μg/ml induced only a slight increase (<10% of the IMP321 effect) of IFN-γ, TNF-α, IL-1β, 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 mAbs, and permeabilized to detect the intracellular expression of TNF-α and CCL4. IMP321, but not the control hlgG1, induced the expression of CCL4 and/or TNF-α in ~5% of PBMCs (Fig. 3, upper panel). Cells expressing both CCL4 and TNF-α 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-α+ cells (black), the single cytokine+ cells (dark gray) and the cytokine- 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-γ, TNF-α, 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+ cells, a subset of NK cells (Fig. 4A, lower panel). A significant increase in the percentage of such cells expressing IFN-γ and/or TNF-α was detected in 52 of 60 donors.

**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 are 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+) were stimulated with or without IMP321 (1 g/ml) and stained with 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+CD56+), the CD8+ NK cells subset (CD3+CD56−CD8+), and the CD8+ T cells subset (CD3+CD56−CD8+). The average values over 60 donors are 1 ± 0.2% and 3.7 ± 0.4% for CD3+CD8+ and CD3+CD56−CD8+ cells, respectively, vs 0.2 ± 0.1% and 0.4 ± 0.004% in medium alone (Fig. 4B, lower panels).

This induction of Tc1 cytokines was also investigated in 21 untreated metastatic cancer patients where an immunosuppressive environment might interfere with the observed effect of IMP321. Because the majority of the healthy subjects were much younger than the cancer patients, only the healthy subjects >40 years old (n = 13) were included in the comparison. The percentages of CD8+ T cells expressing IFN-γ+ and/or TNF-α+ upon IMP321 stimulation were similar (Fig. 4C, left panel) while the percentages of CD8+ NK cells were significantly lower in cancer patients compared with healthy donors (Fig. 4C, right panel).

Similar frequencies were observed for CD3+CD8+TCRγδ+ CD3+CD8+TCRδ+ T cells (data not shown) or for the whole NK population (CD3−CD56+ cells; Fig. 5A). IMP321 also induces the production of CCL4 in a large percentage of CD8+ T cells (>2%) and NK CD8+ cells (>26%) in the three donors analyzed (Fig. 5, B and C). All cells expressing type 1 cytokines are included into the CCL4+ subset (Fig. 5, B and C).

In the intracellular staining assay, neither human IgG1 (n = 8) nor MHC class II mAb (L243 or I3, n = 2) had any effect on expression of intracellular cytokines in CD8+ T cells (Fig. 6A). The Tc1 activation of CD8+ T cells by IMP321 was detectable 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+CD8+ cells could not be induced by IMP321 to produce IFN-γ or TNF-α (Fig. 6C), underlining the role of MHC class II+ 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+ T cells to divide (10 and 8% compared with 4 and 3%, respectively, for the two donors shown in Fig. 6D). 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 CFSElow by the number of cell divisions were 5 and 3% in unstimulated condition compared with 18 and 12% for IMP321-stimulated CD8+ T cells. The same proliferative effect of IMP321 was also observed on CD4+ T cells but to a lesser extent (data not shown).

The IMP321-stimulated cells are Ag-experienced effector or effector memory CD8+ T cells

To further characterize the specific population of IMP321-induced IFN-γ+TNF-α+CD8+ 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.
as CD45RA⁺CD11a⁺, CD45RA⁺CD27⁺, or CD45RA⁺CCR7⁻ CD8⁺ T cell subset while representing a third of the unstimulated CD3⁺CD8⁺ population (Fig. 7A). In contrast, effector T cells were stimulated by IMP321, as CD45RA⁺CD11a⁺ or CD45RA⁺CD27⁺ or CD45RA⁺CCR7⁻ cells were overrepresented in the IMP321-induced IFN-γ/TNF-α /CD8⁺ T cell subset compared with the unstimulated CD3⁺CD8⁺ population (Fig. 7A).

We then analyzed the CD45RA/CCR7 phenotype in a larger cohort of 12 donors to present a more representative analysis of the percentages found in the different subgroups. CM phenotype CD45RA⁺CCR7⁻ CD8⁺ T cells were not stimulated by IMP321 because this subset was less represented in the IMP321-induced IFN-γ/TNF-α /CD8⁺ T cell subset while representing a third of the unstimulated CD3⁺CD8⁺ population (Fig. 7A). In contrast, effector T cells were stimulated by IMP321, as CD45RA⁺CD11a⁺ or CD45RA⁺CD27⁺ or CD45RA⁺CCR7⁻ cells were overrepresented in the IMP321-induced IFN-γ/TNF-α /CD8⁺ T cell subset compared with the unstimulated CD3⁺CD8⁺ population (Fig. 7A).

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**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 p72syk as well as the activation of the PI3K/Akt pathway involved in inducing the maturation process of LAG-3-stimulated DCs in vitro.

**FIGURE 7.** The IMP321-stimulated cells are Ag-experienced effector or EM CD8+ T cells. PBMCs were incubated with IMP321 (1 μg/ml) for 18 h in the presence of brefeldin A for the last 16 h. Cells were stained with CD8-allophycocyanin-Cy7, CD45RA-PE-Cy7, and either CD11a-allophycocyanin, CD27-allophycocyanin, or CCR7-Alexa 647 as indicated, fixed, permeabilized, stained with CD3-PerCP-Cy5.5, anti-IFN-γ-PE, and TNF-α-FITC mAbs and analyzed by flow cytometry (A). The percentage of IFN-γ/TNF-α+ in CD3+CD8+ population is shown in unstimulated (upper left panel) and stimulated conditions (upper right panel). The expression of CD45RA and either CD11a, CD27, or CCR7 were analyzed on total CD3+CD8+ in unstimulated condition (left panels) and on IFN-γ+/TNF-α+ CD3+CD8+ (right panels) in stimulated condition (A). PBMCs from 12 subjects were stimulated or not with IMP321 and stained with CD8-allophycocyanin-Cy7, CD45RA-PE-Cy7, anti-CCR7-Alexa 647 mAbs, fixed, permeabilized, and then stained with CD3-PerCP-Cy5.5, anti-IFN-γ-PE, and granzyme A- or B-FITC mAbs (B). PBMCs from six subjects were stimulated or not with IMP321 and stained with CD8-allophycocyanin-Cy7, CD45RA-PE-Cy7, anti-CCR7-Alexa 647 and CD27-PE (C) or CD28-PE mAbs (D), fixed, permeabilized, and then stained with CD3-PerCP-Cy5.5 and anti-IFN-γ-FITC mAbs. The percentage of the different populations based on the expression of CD45RA, CCR7, and granzyme B, CD27 (C), or CD28 (D) in whole CD3+CD8+ population and in CD3+CD8+ expressing IFN-γ was determined in unstimulated (left panels) and IMP321-stimulated (right panels) conditions, respectively. The means of percentage of each population are presented on statistical views. M, memory; N, naive; E, effector.
These observations were made on healthy subject PBMCs and it is of 6-sulfo LacNAc-expressing DC (21, 22), and monocytes. IFN-γ known to be directly cytotoxic for tumor cells when activated by IFN-γ mined by flow cytometry. The percentage of IFN-γ-expressing CD8+ T cells that were fully differentiated, as shown by the lack of CD27 and CD28 expression (29), were driven by IMP321 into Th1/Tc1 differentiation, and antitumor cytotoxicity (30, 32–34). Antitumor cytotoxicity refers to the ability of immune responses to tumor cells. The clinical development of non-TLR ligands such as IMP321 may be used to recruit a large range of effector cells in both innate and acquired immune 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...