Poly-functional and long-lasting anticancer immune response elicited by a safe attenuated *Pseudomonas aeruginosa* vector for antigens delivery

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Live-attenuated bacterial vectors for antigens delivery have aroused growing interest in the field of cancer immunotherapy. Their potency to stimulate innate immunity and to promote intracellular antigen delivery into antigen-presenting cells could be exploited to elicit a strong and specific cellular immune response against tumor cells. We previously described genetically-modified and attenuated *Pseudomonas aeruginosa* vectors able to deliver in vivo protein antigens into antigen-presenting cells, through Type 3 secretion system of the bacteria. Using this approach, we managed to protect immunized mice against aggressive B16 melanoma development in both a prophylactic and therapeutic setting. In this study, we further investigated the antigen-specific CD8+ T cell response, in terms of phenotypic and functional aspects, obtained after immunizations with a killed but metabolically active *P. aeruginosa* attenuated vector. We demonstrated that *P. aeruginosa* vaccine induces a highly functional pool of antigen-specific CD8+ T cell able to infiltrate the tumor. Furthermore, multiple immunizations allowed the development of a long-lasting immune response, represented by a pool of predominantly effector memory cells which protected mice against late tumor challenge. Overall, killed but metabolically active *P. aeruginosa* vector is a safe and promising approach for active and specific antitumor immunotherapy.

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OSTAB attenuated strain is deleted for major T3SS-exotoxins ExoS and ExoT and for two subunits (UvrA and UvrB) of the exonuclease ABC, which takes part in the nucleotide excision repair (NER) pathway that removes DNA damage induced by ultraviolet (UV) light. Following photo-inactivation, with long-wave UV light (UVA) radiation associated to the DNA crosslinking agent amotosalen (psoralen S-59), the bacteria become unable to replicate and are doomed to die, but are still immunologically and T3SS active. Thus, after photo-inactivation, the OSTAB bacteria turn into killed but metabolically active (KBMA). This strain is able to deliver any tumor-associated antigen of interest into host cells through T3SS and to drive cellular immune response against the tumor. We have previously shown that OSTAB attenuated and KBMA strains trigger antigen-specific CD8+ T cell priming, yielding to the protection of mice against aggressive melanoma development. In order to further characterize the Pa-induced antitumor responses, we have investigated the phenotype, the functionality, and the persistence of Pa-induced antigen specific CD8+ T cell response. We demonstrate that multiple immunizations with a safe attenuated KBMA Pa aeruginosa vector induce a highly functional antigen-specific cellular immune response able to control B16 tumor growth in mice with a similar efficiency as the OSTAB attenuated Pa vector. Interestingly this was associated with a significant increase of antigen-specific tumor-infiltrating lymphocytes (TILS) and a benefit ratio antigen-specific effector CD8+ T cells/regulatory T cells within the tumor bed. Finally, we show that a pool of predominantly effector-memory antigen-specific CD8+ T cells persists after immunizations and confers a long term protection against tumor development.

RESULTS
KBMA vector based vaccine induces highly functional cellular immune response

We have previously shown the ability of KBMA Pa at inducing antigen-specific CD8+ immune response in vivo and to prolong survival of mice challenged with tumors. We aimed here at analyzing more deeply the efficiency of this vaccine. First, we focused on an accurate analysis of the functionality of the antigen-specific CD8+ T cells generated after immunizations. Thus the established immunizations schedule was applied (6 subcutaneous immunizations, 2×/week). We then assessed the frequency and the functionality of splenic Ova tumor-specific CD8+ T cell 10 days after last immunization with attenuated OSTAB S54-OVA or KBMA S54-OVA vaccine. Both approaches (OSTAB S54-OVA and KBMA S54-OVA) were able to induce the priming of tumor-specific T cells at similar frequencies (11.9 and 12.1%, respectively) (Figure 1a). To evaluate their functionality, cytokine secretion and expression of the degranulation marker (CD107a) were investigated after splenocytes stimulation with the immune-dominant MHC class I restricted OVA peptide (SIINFEKL epitope). Similar cytokine profiles were observed on tumor-specific CD8+ T cells following peptide stimulation in both vaccinated groups (Figure 1b–d). Tumor-specific CD8+ T cell response induced by OSTAB S54-OVA and KBMA S54-OVA vaccines displays a high degree of polyfunctionality, with respectively 12% and 11% of responding CD8+ T cells producing at the same time interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IL-2 in association with degranulation and 58 and 52% respectively, producing at least two cytokines with degranulation (Figure 1c,d). Single functional specific CD8+ T cell represented a minority with only 13% in OSTAB S54-OVA and 12% in KBMA S54-OVA groups. These results are consistent with our previous observations that KBMA and OSTAB attenuated Pa-OVA vectors have similar ability at inducing potent cellular antitumor immune response.

Pa vectors based vaccines induce strong antigen-specific CD8+ T cells tumor infiltration and a positive shift between effector and immunosuppressive T cells

The efficacy of KBMA vaccine has been evaluated in a therapeutic setting. B16-OVA-bearing mice received up to five injections starting when tumors became palpable (Figure 2a). While mice that received control OSTAB S54-Ei (Pa strain without antigen) or heat-killed (HK) S54-OVA (dead strain unable to deliver antigen through T3SS) develop tumors over time, KBMA S54-OVA vaccine allows a significant control of tumor progression, comparable to live OSTAB S54-OVA strain (Figure 2b,c). More importantly, tumor growth curves reveal that KBMA S54-OVA treated mice have homogeneously responded to the treatment (Figure 2c). A mild therapeutic and heterogeneous effect was also observed with HK S54-OVA treatment compared with the OSTAB S54-Ei negative control. This result was expected since the HK S54-OVA immunization condition is closed to adjuvanted whole protein vaccination strategies that have shown such a mild protection effects in the clinic.

Many studies have reported that high level of CD8+ TILs strongly predicts patient overall survival undergoing the critical role of tumor-specific CD8+ T cells. However, one challenge in cancer vaccine development is not only to induce a strong CD8+ T cells effectors infiltration within the tumor, but also to overcome the immunosuppressive environment by shifting the balance between effector cells and regulatory cells. The analysis of TILs, 21 days posttumor implantation, revealed that both live and KBMA Pa vectors induce a significant increase in the Ova-specific CD8+-T cells within the tumor while empty or HK vectors failed to do so (Figure 3a,b). Importantly, both bacterial vectors induce a strong increase in the tumor-specific CD8+-T cells/T-regs ratio, thus positively shifting the balance between effector and immunosuppressive T cells (Figure 3c).

Pa vectors elicit a stable pool of tumor-specific CD8+ T memory cells

A key feature of the efficacy of a cancer vaccine lies on its ability to induce a long-lasting tumor-specific CD8+ T cells response and subsequent protection against relapse and/or metastasis. Thus, we analyzed the potency of Pa vaccination by the evaluation of the development of tumor-specific CD8+ memory response during and after six consecutive immunizations (2×/week). As soon as 6 days after the first immunization, tumor-specific CD8+ T cells in the blood were detected in groups treated with OSTAB S54-OVA or KBMA S54-OVA, reaching 2.2 and 1.8% respectively among CD8+ T cells as measured by multimers whereas in control groups HK S54-OVA or OSTAB S54-Ei empty vectors only 0.1% tetramer positive cells among CD8+ T cells corresponds to background signal (Figure 4a). In both, OSTAB S54-OVA or KBMA S54-OVA treated groups we observed an increase of tumor-specific CD8+ T cells number during the immunization period, albeit in a lesser extent in KBMA treated group (Figure 4a). The maximal number of tumor-specific CD8+ T cells was reached around 1 week after the last vaccination. This phase was followed by a biphasic contraction period, where the number of tumor-specific CD8+ effector T cells decreased in the two groups during 2 weeks after the last vaccination and then stabilized during next month. At 70 days, a substantial persisting pool of tumor-specific memory CD8+ T cells in both treated groups was still observed.
To study the phenotypic changes of blood circulating tumor-specific CD8+ T cells over time, we followed the expression of CD127 (IL-7Rα) memory marker,22 homing receptor CD62-L and the killer cell lectin-like receptor G1 (KLRG-1) on antigen-experienced T cells23 (OVA specific) (Figure 4b). After the first immunizations (at day 6), tumor-specific CD8+ T cells from OSTAB S54-OVA and KBMA S54-OVA group had a typical effector phenotype CD127 low CD62-L low KLRG-1 low (TEFF). Then, specific CD8+ T cells increased CD127 memory marker expression (from day 21) but kept a predominant effector phenotype (CD62-L low), with a progressive increase of KLRG-1.

Figure 1
Functional characterization of splenic Ova-specific CD8+ T cells response 10 days after last immunization. (a) Representative flow cytometric profile (up) and frequency (down) of Ova-specific CD8+ T cells evaluated by H2Kb-SIINFEKL multimer staining on total CD8+ gated cells (b) Simultaneous production of the cytokines IFN-γ, TNF-α, and IL-2 by CD8+ T cells as well as degranulation (CD107a) marker were assessed during 4 hours OVA peptide (SIINFEKL) stimulation or in absence of stimulation (Medium). (c) Frequency of total CD8+ T cells allocated in each of the 15 analyzed combinations of IFN-γ, TNF-α, IL-2, and CD107a marker according to the diagram shown underneath the bar graphs. (d) Pie charts represent the spectrum of functions (cytokines and/or degranulation marker) among responding CD8+ T cells to OVA peptide stimulation, in KBMA and OSTAB groups. Each slice of pie represents the percentage of cells expressing between 1 and 4 effector functions. Mean ± SEM. IFN-γ, interferon-γ; IL-2, interleukin-2; KBMA, killed but metabolically active; TNF-α, tumor necrosis factor-α; SEM, standard error of the mean.
expression (day 21, 28, and 56). Remarkably expression of this T cell senescent marker is corroborated with the drop of tumor-specific blood CD8+ T cells numbers from day 28 (Figure 4a). At 71 days after the first immunization a circulating pool persists comprising both effector (CD62-Llow CD127low) and central-memory cells (CD62-Lhi CD127hi) in OSTAB S54-OVA and KBMA S54-OVA group with KLRG1hi and KLRG-1low cells.

Finally, we also investigated the tumor-specific CD8+ T cell response in splenocytes at effector phase (day 29) and during memory phase, this tumor-specific CD8+ T cells percentage of TEM cells were measured with OSTAB S54-OVA group with 9% versus the KBMA S54-OVA (51.8% versus 68.2%, P < 0.0001). As a consequence a lower T cell senescent marker is corroborated with the drop of tumor-specific blood CD8+ T cells expression (day 21, 28, and 56). Remarkably expression of this T cell senescent marker is corroborated with the drop of tumor-specific blood CD8+ T cells numbers from day 28 (Figure 4a). At 71 days after the first immunization a circulating pool persists comprising both effector (CD62-Llow CD127low) and central-memory cells (CD62-Lhi CD127hi) in OSTAB S54-OVA and KBMA S54-OVA group with KLRG1hi and KLRG-1low cells.

Tumor-specific CD8+ T memory cells induced by Pa-antigen vaccines promote protective long-term antitumor response
Since a remarkable stable memory CD8+ T cell response was reached ~50 days after the last immunization in mice, we challenged immunized mice by B16-OVA tumor cell implantation 100 days after the last immunization with P. aeruginosa vectors (Figure 5a). Mice immunized by OSTAB S54-OVA and KBMA S54-OVA vector remained tumor free beyond 150 days post-tumor challenge in 67% and 57% respectively of all individuals. At the opposite, mice from control groups HK S54-OVA and OSTAB S54-Ei develop tumors and had a median survival of 29 and 22 days, respectively (Figure 5b). We observed significantly better survival in mice vaccinated with OSTABSS54-OVA and KBMA S54-OVA compared with control mice (strain OSTAB S54-Ei) (The P-value for the log-rank test are 0.0096 and 0.0005 respectively) whereas there is no statistical difference between OSTAB S54-OVA and KBMA S54-OVA (P = 0.9375). Thus, vaccination with P. aeruginosa vectors induces a long term protection and confers a great benefit to efficiently immunized mice.

DISCUSSION
In this study, we show that attenuated OSTAB S54-OVA and KBMA S54-OVA elicit a similar polyfunctional cellular antitumor immune response. Then, using B16-OVA tumor model, we further characterize T CD8+ antitumor immune response following immunization with both Pa-OVA vectors in terms of tumor infiltration and long-term protection. First, we demonstrate that we significantly inhibit tumor growth in a therapeutic setting, for both groups in this aggressive tumor model. This tumor growth control is correlated with a strong tumor infiltration by tumor-specific CD8+ T cells.
Furthermore, we show that the antigen-specific response is also accompanied by a significant rise of the proportion of antigen-specific CD8+ T cells among TILs and accordingly a shift in the balance T CD8+/CD4+Treg in favor to effector CD8+ cells. This observation is consistent with previous data from either mouse models or clinical trials describing that shifting the balance toward CD8+ T cells versus Treg is critical for effective cancer immunotherapy. Live-attenuated and KBMA Pa-OVA vectors, as for KBMA Lm, induce systemic T-cell responses including polyfunctional cytokine-secreting CD8+ T-cells.\textsuperscript{24} The contribution of different subsets of memory CD8+ T cells in recall responses following immunization with live attenuated bacterial vectors are poorly understood and still controversial. In this study, we explored the antigen-specific CD8+ memory response induced by Pa-OVA vectors and we show that antigen-specific CD8+ T cells evolve toward a pool of effector- and central-memory phenotype. This long-lasting response is correlated to a potent protection of mice against tumor challenge at the memory phase. The memory pool of CD8+ T cells, identified by its re-expression of CD127 (IL-7Rα) after effector phase,\textsuperscript{25} contains two major distinct subsets, the so-called T effector-memory (TEM) and T central-memory (TCM), that differ in their phenotype, trafficking patterns, tissue residence and functional characteristics.\textsuperscript{26–28} We distinguished a difference in the memory phenotype of tumor-specific CD8+ T cells induced by the two vectors. It seems that KBMA Pa confers a strong memory response (TCM and TEM) whereas OSTAB-S54 OVA strain elicit an extended effector phenotype (~26% of cells versus 9% for KBMA bacteria), even at the memory phase despite the initial CD8+ T-cell response being stronger in tumor challenge after immunization with KBMA Pa-OVA, even at the memory phase.

Figure 3  Immunizations with OSTAB S54-OVA or KBMA S54-OVA vaccines allow Ova-specific CD8+ T cells tumor infiltration and change the balance between effector and regulatory T cells within the tumor. Mice were immunized according to the schedule depicted in Figure 2a. Tumors were harvested at day 21. (a and b) Ova-specific CD8+ T cell tumor infiltration quantified by flow cytometry in dissociated tumors. (a) Representative flow-cytometric profile of each group indicating percentage of Ova CD8+ T cells among CD8+ TILs. (b) Mean percentage of Ova CD8+ T cells through CD8+ TILs. (c) Ratio between Ova CD8+ T cells (gated on CD8+ TILs) and Treg cells (% FoxP3+ cells gated on tumor CD4+ cells). n = 4–6 mice/group. Data are representative of two pooled experiments. Mean ± SEM. **P < 0.01, ***P < 0.001 determined by unpaired Student’s t-test. KBMA, killed but metabolically active; TILs, tumor-infiltrating lymphocytes; SEM, standard error of the mean.
Figure 4  Kinetic of Ova CD8⁺ T cells response and memory markers induced by Pa-OVA vaccines analyzed by flow cytometry in spleen and blood. Mice received 6 immunizations with Pa-OVA vaccines or controls (first immunization at D0) and blood or spleens were harvested at different time point. (a) Representative flow-cytometry profiles of Ova-specific CD8⁺ T cells in blood for treated and control groups at day 6 (up). Kinetic profile of Ova-specific CD8⁺ T cells (absolute number) in the blood (down). n = 5 or 6 mice per group per time point. Mean ± SEM. (b) Expression of CD62-L, CD127, and KLRG-1 markers assessed over the time by gating on Ova-specific CD8⁺ T cells in blood. (c) Representative profile of CD62-L and CD127 markers coexpression by gating on Ova-specific CD8⁺ T cells in splenocytes at effector phase (day 29) and memory phase (day 90). Numbers indicate percentage within each quadrant (mean ± SEM). n = 5 or 6 mice (left). Mean percentage of Memory (CD62-Llo CD127hi or CD62-Lhi CD127hi) and effector (CD62-Llo CD127lo) Ova-specific CD8⁺ T cells subpopulations at 90 days in splenocytes upon immunization with Pa-OVA vaccines. TEM: effector memory Ova-specific CD8⁺ T cells; TCM: central memory Ova-specific CD8⁺ T cells; TEFF: OVA-specific effector CD8⁺ T cells. Mean ± SEM. *P < 0.05, ***P < 0.001 determined by unpaired Student’s t-test. Pa, Pseudomonas aeruginosa; SEM, standard error of the mean.
antigen delivery demonstrated their attractive properties.30–32 They tumor-of investigation to improve the quality of the antitumor immune in vivo limited response against the tumor growth. It seems that the modu-
this fundamental step. Nevertheless, many of them induced only a response(s). Several strategies, like adjuvanted peptide-based vac-
protection correlated to disparate CD8+ memory response follow-
previous preclinical study has described distinct long-lasting tumor T cells (CD62-Llow CD127high) that were unable to expand and led to a
memory phase. This could be explained by the presence of remain-

Figure 5 Long-term protection induced by Pa-ova vaccines against
B16-OVA melanoma. (a) Experimental setting. Mice have been injected
6 times (2x/week) and challenged with 2 x 10^6 B16-OVA cells 100 days
after the last immunization. Tumor growth was monitored every 2
days and mice were killed when tumor reached 150 mm^3. (b) Percent survival
(based on 150 mm^3 tumor surface) of mice challenged with tumors at
memory phase. n = 5–7 mice per group. Log-rank test OSTAB S54-ova
and KBMA S54-OVA compared with OSTAB S54-Ei P = 0.0096 and P =
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Material and methods
bacterial strains, cell line, and mice
the attenuated Pa OSTAB mutant susceptible to photochemical treat-
ment and the corresponding OSTAB S54-Ei ("empty" plasmid with ExoS_m
but without antigen) and OSTAB S54-OVA (chicken ovalbumin D248-A376
antigen fused to ExoS_m) transformed strains were previously described.31
Transformed bacteria were grown in lysogenic broth (LB) (Sigma-Aldrich,
St Louis, MO) medium in the presence of 300 mg/l carbenicillin at 37°C
and 250g.

B16-OVA melanoma cells from C57Bl/6 mice were cultivated in complete
Dulbecco’s modified essential medium (DMEM) Glutamax containing 10%
fetal bovine serum (FBS), 100 U/ml of penicillin and 100 mg/ml of strepto-
mycin, and 500 µg/ml Geneticin. The cells were passaged using 0.05% tryp-
in/0.02% ethylenediaminetetraacetaete (EDTA) solution and were cultivated
at 37°C in a 5% CO2 humidified incubator. Medium and reagents were pur-
chased from Thermo Fisher Scientific, Waltham, MA.
Female C57Bl/6J mice were purchased at 5–6 weeks old from the Elevage
Janvier (Le Genest St. Isle, France) and were kept under specific pathogen-
free conditions in an accredited establishment (PHTA animal facility, Plateforme
de Haute Technologie Animale) according to the governmental guide-
lines N886/609/CEE. All animal experiments were approved by the Animal
Experiment Committee of the Region (Cometh) and submitted to the French
Ministry of Research and received Reference Number 200452.02 for the protocol
entitled “Characterization of immune response induced by a bacterial vector for
murine tumor”. Mice were anesthetized with isoflurane prior to subcutaneous
(s.c.) bacteria injection and for retro-orbital blood sampling.

Photochemical treatment for KBMA Vaccine
KBMA bacteria were prepared as previously described.30 Briefly, OSTAB S54-
OVA strain was inoculated in LB at OD_{600} 0.2 with 1 mmol/l of isopropyl-
β-D-thiogalactoside (IPTG) (Sigma-Aldrich) and 300 mg/l of carbenicillin. When the OD_{600}
reached 0.5, 10 µmol/l of photoactivatable amotosalen intercalant
was added. Then cultures were grown until OD_{600} ≥ 1. About 1 ml of cul-
ture was then transferred in a well (0.6 cm^2) of a six-well cell culture plate
for UVA irradiation at a dose of 7.2 J/cm^2 with a Stratalinker 1800 device
(Stratagen, La Jolla, CA). Viability of the photochemically inactivated cultures
was assessed by plating bacteria on Pseudomonas Isolation Agar (PIA) and
counting of the colony-forming units. Following the photochemical treatment
with 10 µmol/l of amotosalen one live organism per 1.25 x 10^{6} bacteria
is able to replicate after OSTAB strain photo-inactivation.

Immunizations
For immunizations, KBMA bacteria were prepared as described above. Live
OSTAB S54-ova and OSTAB S54-Ei strains were grown overnight in LB con-
taining 300 mg/l of carbenicillin at 37°C and 250rpm. The next day, after two
washing steps in LB, bacteria were resuspended in LB supplemented with 1
mmol/l IPTG and 300 mg/l of carbenicillin and at OD_{600} 0.2 and cultured until
an OD_{600} = 1.6 (~3 hours of culture). Then bacteria (KBMA and live OSTAB
strains) were harvested and washed two times in phosphate buffered saline

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Germany) was performed and samples were then incubated for 30 minutes to exclude dead cells for flow cytometry analysis. Flow cytometry acquisition was done using a BD Biosciences FACSCanto II and analysis carried out with FlowJo software (Tree Star, Ashland, OR).

CD8+ T cell function and intracellular staining

For the tumor infiltration study, cells were stained first with H2Kb-SIINFEKL Dextramer, a multimer of H2Kb loaded with OVA257-264 antigen was purchased at Immudex (Copenhagen, Denmark). Staining was performed as described by manufacturer’s instruction. The Fixable Viability Dye (FVD eFluor780, eBioscience) was used for tumor-infiltrated study in order to exclude dead cells for flow cytometry analysis. Flow cytometry acquisition was done using FlowJo software (Tree Star, Ashland, OR).

Phenotypic characterization of antigen-specific CD8+ T cells

Splenocytes or blood cells were harvested at various time intervals, stained first with H2Kb-SIINFEKL multimers and with antibodies against CD45 (only for blood cells), CD4, CD8, CD44, CD127, CD62L, and KLRC1. For absolute counting in blood samples, we used Flow-Count Fluorospheres (Beckman Coulter, Miami, FL). Blood cells were fixed in fluorescence-activated cell sorting (FACS) lysing buffer (BD Biosciences) and acquired by flow cytometry. For the tumor infiltration study, cells were stained first with H2Kb-SIINFEKL multimers, then with antibodies against CD45, CD4, CD8, and CD44, and with the fixable viability dye. In a second step, intracellular staining of FoxP3 was performed with the FoxP3/Transcription Factor Staining Buffer Set (eBioscience).

CD8+ T cell function and intracellular staining

Intracellular cytokines production by antigen-specific CD8+ T cells was carried out by 4 hours restimulation with splenocytes in the presence of SIINFEKL peptide (OVA257-264 10 µg/ml) or without peptide as a control. Monensin (0.7 µl/ml, eBioscience) and anti-CD107a antibody to analyze degranulation by cytotoxic T lymphocytes were added before starting restimulation. Cells were then surface-labeled with anti-CD8, anti-CD4 antibodies and were subjected to intracellular cytokines staining (BD Biosciences). Intracellular cytokines were analyzed only on CD8+ T cells.

Statistical analysis

Results are expressed as the mean ± SEM unless stated otherwise. Statistical comparisons between two groups were evaluated by the Student’s t-test. A P-value < 0.05 was considered to indicate statistical significance. Unless otherwise indicated, all experiments were conducted at least twice. Log-rank tests ( Mantel-Cox) were used to compare the Kaplan–Meier tumor survival curves. Statistical and graphical analyses were performed using GraphPad Prism Version 6 (GraphPad Software, La Jolla, CA).

CONFLICTS OF INTEREST

D.H., A.L.G., B.P., L.B., and B.T. declare competing financial interest. A.L.G., B.P., and L.B. are the cofounders of APCure, a start-up that produces immunotherapy treatments for patients with cancer.

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