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Engineered CCR5 superagonist chemokine as Adjuvant in anti-tumor DNA vaccination

Karim Dorgham^{†,‡}, **Valérie Abadie^{†,‡}**, Mutsunori Iga^{†,‡}, Oliver Hartley[¶], Guy Gorochov^{+,†,‡,¶},
Behazine Combadière^{+,†,‡}

[†]Institut National de la Santé et de la Recherche Médicale (INSERM) U543, [‡]Université Pierre et Marie Curie (**UPMC Univ Paris 06**), 91 boulevard de l'hôpital, 75634 Paris, France, [#]Immunologie A, Assistance Publique Hôpitaux de Paris (AP-HP) Hôpital Pitié-Salpêtrière, 83 boulevard de l'hôpital, 75634 Paris, France and [¶]Département de Biologie structurale et Bioinformatique, Centre Médical Universitaire, 1 rue Michel Servet, 1211 Geneva 4, Switzerland.

⁺**Correspondence should be addressed to:** Behazine Combadière: combadie@ccr.jussieu.fr or to Guy Gorochov: guy.gorochov@psl.ap-hop-paris.fr
Laboratoire d'Immunologie Cellulaire, INSERM U543, **UPMC Univ Paris 06**, 91 bd de l'hôpital, 75634 Paris Cedex 13, France. Tel: +33140779888; Fax: +33140779734

Abstract

Chemokine receptors are promising targets for enhancing T cell immunity and anti-cancer therapy. CCL5 is a potential adjuvant for DNA vaccination. We postulated that CCR5 superagonists could be even more effective. A CCR5 superagonist derived from natural CCL5 by directed *in vitro* evolution, namely 1P7, is used as a DNA vaccine adjuvant and expressed as fused chemokine-Ig (1P7-Ig). We show that OVA+1P7-Ig DNA co-inoculation induced higher frequencies of OVA-specific CD8 lymphocytes than OVA+CCL5-Ig or controls and gave an even better protection against tumor growth in a CCR5-dependant manner. Our results indicate that CCR5-superagonists may provide potent adjuvants for vaccines.

Key words: CCR5 superagonist, DNA vaccine, genetic adjuvant, tumor vaccine.

Abbreviations: APC, antigen presenting cell; OVA, Ovalbumin; DC, dendritic cell; CCL, CC chemokine ligand; CCR, CC chemokine receptor

1. Introduction

CCL5 is a small 68 amino acids protein produced predominantly by CD8⁺ T-cells, epithelial cells, fibroblasts and platelets [1]. Besides its role in facilitating the deployment of antigen-presenting cells (APC) and immune cells (NK, B- or T-cells), CCL5 is clearly associated with Th1-type cellular responses [2, 3]. Therefore, CCL5 plays a key role in the induction of adaptive immune responses [4]. In humans, the presence of intratumoral T cells correlates with improved clinical outcome in advanced ovarian carcinoma [5] and in colorectal cancer [6, 7]. Accumulating evidence in mice [8-10] and in humans [5, 6, 11] suggests that this protective anti-cancer response is mediated in part by effector T cells and long-lived memory T cells [7]. Thus, improvement of the intensity of the effector and memory T cell response might have an important impact in the control of tumor growth.

DNA vaccination is an attractive approach for cancer immunotherapy [12, 13], however one major drawback is its limited potency. We and others have shown that plasmid-encoded cytokines, chemokines, or co-stimulatory molecules are promising DNA vaccine adjuvants against viral infections [14-17]. Similar approaches using chemokines such as CCL19 and CCL21 as adjuvant for DNA vaccination have been also proposed to enhance anti-tumor immunity [18-20]. In a preventive DNA vaccination model, we have recently shown that CX3CL1-Ig can enhance tumor-antigen specific T cell priming after a single DNA injection and protect against tumor growth [17]. In addition, in a therapeutic tumor model when the tumor is installed, intratumoral DNA injection coding CCL5-Ig reduces tumor growth, based on intra-tumoral recruitment of immune cells (DC, NK, CD4 and CD8 cells) in both an antigen-specific and non-specific manner suggesting the involvement of both adaptive and innate immunity [21]. Previous studies have explored the use of CCL5 as a genetic adjuvant for DNA vaccines that encode antigens derived from various pathogens such as HIV-1 [22, 23], HPV [24] or HSV [25] in preventive vaccination, however several DNA

injections are necessary for vaccine efficacy. These studies all showed that exogenous CCL5 is effective in amplifying the adaptive branch of the immune response to DNA vaccines, as documented by enhancement of Ag-specific T helper cell proliferative or CTL responses, and increased Th1 cytokine (IFN-gamma and TNF-alpha) production. CCL5 induces leukocyte migration by binding to specific receptors in the seven-transmembrane G-protein coupled receptor family, CCR1 [26], CCR3 [27] and CCR5 [28]. CCL5 influences the trafficking of both T-cells prevalently associated with Th1 responses [29] and of effector T cells [30, 31]. It also acts on a range of other cells, including basophils, eosinophils, natural killer (NK) cells, monocytes, dendritic cells (DCs) and mast cells [32]. CCL5 production is induced by the combination of Th1 type cytokines (TNF-alpha, IFN-gamma) and inhibited by Th2 type cytokines (IL4 and IL13) [33]. At this stage, we postulated that mutant chemokine that behave as CCR5 superagonists might be even more effective than wild type CCL5 at amplifying the adaptive immune response.

We have previously reported the construction of phage chemokine libraries and the identification of CCL5 analogues with increased capacity to engage CCR5, including a superagonist [34]. Subsequent work using this approach led us to identify a new mutant; 1P7. The present work described for the first time a new CCR5 superagonist and its potential efficacy in DNA vaccination.

We designed a 1P7-immunoglobulin (1P7-Ig) expression plasmid to be co-inoculated with the anti-tumor DNA vaccine based on our previous work [17]. The chemokine-Ig fusion was used because it presents the advantages of divalent affinity, non cytolytic effect and long *in vivo* half-life with conserved activity of both proteins [21, 35]. Here, we show that compared to Ig fused to wild type CCL5, 1P7-Ig significantly enhanced effector CD8 T cell anti-tumor response.

2. Materials and Methods

2.1. Mice, cell lines and chemokines

Wild-type C57BL/6 mice were purchased from Elevage Janvier (Le Genest, Saint Isle, France) and C57BL/6 CCR5 ^{-/-} mice from Jackson Laboratories (Charles River, Saint-Aubin les Elbeufs, France). All mice were females of 6 to 10 weeks old. All experiment protocols were approved by the local animal experimentation and ethics committee guidelines.

Tumor cell lines derived from C57BL/6 mice were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The H-2b lymphoma EL4 and its derivative E.G7-OVA, which expresses chicken ovalbumin (OVA), were maintained as described previously [21]. CHO-CCR5, HEK-CCR5, HEK-CCR1, **HEK-CCR3** and HeLa-CD4-LTRLacZ cells stably expressing CCR5 (HeLa-P5L) have been described previously [34]. CCL5 analogues proteins were prepared by total chemical synthesis, as described elsewhere [36] and amino acid sequences are presented in Table 1.

2.2. Calcium Mobilization Assay

Experiments were carried out essentially as in [34] using a FLEX-station machine (Molecular Devices, CA, USA). HeLa-P5L cells, HEK-CCR1 **or HEK-CCR3** cells were loaded with Fluo-4 (Invitrogen, Molecular Probes, France) according to the manufacturer's recommendations and maintained at 37°C. Peak signals were defined as the area under the trace (Calculated using SoftMax Pro software (Molecular Devices) and expressed in relative fluorescence units) during a 45 second period following chemokine addition. Determinations were performed in duplicate and dose-curves were fitted using Prism software (Graphpad Software, San Diego, CA, USA).

2.3. CCR5 receptor down-modulation experiments

HEK-CCR5 cells (10^5) were incubated for 30 min at 37°C in supplemented culture medium containing various concentrations (0 to 100 nM) of chemokines. Medium alone was used for controls. Cells were washed 5 times with cold PBS and incubated for 30 min on ice with 0.5 µg of anti-CCR5 mAb (clone 2D7 phycoerythrin-conjugated, PharMingen, France). Cells were washed two times, fixed with 4% paraformaldehyde, and analyzed with a FACScalibur using Cell Quest software (Becton Dickinson, San Diego, CA, USA). At least 10,000 events were accumulated for each sample.

2.4. Competitive radio-ligand binding on CCR5

Experiments were performed as in [37]. CHO-CCR5 cells were seeded into 24 well plates (1.2×10^5 cells/well). After overnight incubation, competition binding was performed on whole cells for 3 h at 4°C using 12 pM radiolabeled CCL3 (Amersham, France) plus variable amounts of unlabelled ligand. Determinations were made in quadruplicate and IC₅₀ values were derived from monophasic concentration inhibition curves fitted using Prism software.

2.5. Plasmid construction

Expression vector coding chicken ovalbumin (OVA) was prepared as previously described. Briefly, OVA ORF encoding amino acids 1-386 (GeneBank # J00895) was prepared by polymerase chain reaction (PCR) from cell line E.G7-OVA cDNA and subcloned into the plasmid vector pVRC (kindly provided by Dr. Barouch,; Division of Viral Pathogenesis, Beth, Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA). The chimeric CCL5-Ig expression vector used was as previously described [21]. As with the CCL5-Ig construct, we used the Fcγ2a fragment

mutated in the Clq binding motif (E318, K320, K322) and the FcγRI binding site (L235) to produce a noncytolytic form of 1P7-Ig [14, 35]. The 1P7 DNA sequence was amplified from the phage display vector using a specific upstream primer encoding the human CCL5 signal peptide 5'-TTT CTG CAG ATG AAG GTC TCC GCG GCA CGC CTC GCT GTC ATC CTC ATT GCT ACT GCC CTC TGC GCT CCT GCA TCT GCC ATG TCC CCA CTG TCC-3' (the recognition site for *PstI* is underlined) and a downstream primer 5'-CAA CTC TTT GGA GAT GTC TAG AGG GGC CCC A-3' (the recognition sites for *XbaI* and *PspOMI* - in that order- are underlined). The product was cut with *PstI* and *PspOMI* and inserted into pVRC that had been linearized using *PstI* and *NotI*. The ligated DNA was electroporated into *E. coli* TG1 and DNA inserts of selected clones were sequenced using an automatic sequencer ABI 377 (Perkin Elmer, USA). All plasmids were produced by Tebu-Biolab (Tebu, France) in endotoxin-free conditions.

2.6. DNA vaccination and mice immunomonitoring

Three groups of mice were immunized intramuscularly by co-administration of 100 µg OVA plasmid DNA and 100 µg of one of the following: Ig plasmid DNA, CCL5-Ig plasmid DNA or 1P7-Ig plasmid DNA. Blood samples were collected in RPMI 1640 containing 40 U/ml heparin by retro-orbital puncture before each immunization (day 0) and on day 15. Previous experiments showed that day 15 is the peak of OVA-specific T cell detection in blood after DNA immunization [17]. Following red blood cells lysis, cell suspensions were stained with PE-conjugated H-2Kb/OVA 257-264 (SIINFEKL) tetramer (Proimmune Oxford, UK) and anti-CD8-APC monoclonal antibody (clone Ly-2; Becton Dickinson, France). Samples were washed, fixed and **ten thousand CD8 events were** analyzed by flow cytometry with CellQuest™ software.

2.7. Tumor challenge

Three groups of mice were injected subcutaneously in the right flank with 2×10^4 tumor cells in 100 μ l PBS. Tumor size was measured three times a week with a caliper, and the tumor volume was estimated from the following formula: width x length x (width + length)/2.

2.8. *In vivo* cytotoxic T cell assay

Detection of *in vivo* cytotoxic T lymphocytes (CTL) was performed as described previously [38]. Splenocytes were prepared from naïve animals by passing through sterile meshes. Erythrocytes were removed using red-blood cells lysis buffer for five minutes followed by three washes in PBS 1X and resuspending at 5×10^6 cells/ml. Cells were divided into two populations, one of which was pulsed with 1 μ g/ml of SIINFEKL (MHC-Class I restricted peptide) for 90 min at 37°C. Cells were then washed and labeled with CFSE (Molecular Probes, Eugene, OR) for 15 min at 37°C. Peptide-pulsed cells were labeled with 5 μ M of CFSE (CFSE^{high}) and unpulsed cells with 0.5 μ M (CFSE^{low}). Cells were then washed once in PBS-2% FCS and twice in PBS 1X prior to intravenous injection into recipient mice. Cells were recovered 20 h after transfer for flow cytometric analysis using CellQuest software (Becton Dickinson Immunocytometry Systems, san José, CA). One million live events were acquired for further CFSE analysis.

2.9. Data analysis

Statistical analysis for comparison of two groups of mice were performed using the unpaired Student's *t*-test and Graphpad software. Data handling and presentation were performed using Prism4.0 software. Probability values (P) < 0.05 were considered significant.

3. Results

3.1. Chemokine variant 1P7 is a CCR5 superagonist.

In a preliminary screen of a panel of CCL5 mutants isolated from our earlier phage display work on CCR5 [34], 1P7 was identified as a strong CCR5 agonist. Comparison with native CCL5 shows 1P7 to be a CCR5 superagonist, giving a maximum response ($E_{\max}$) at 170% of that of CCL5, and capable of eliciting CCR5 signaling at concentrations where CCL5 gives no detectable signal (Fig 1A). We then compared the capacity of 1P7 with that of the native CCL5 to elicit CCR5 down-regulation. Under the assay conditions used, 1P7 induced significant dose-dependent down-modulation of CCR5 with a response at 1 nM compared to that of 100 nM CCL5 (Fig 1B). These observations are in agreement with our previous work, which showed that CCR5 down-modulation is closely linked with receptor activation [34]. The increased signaling activity is not a consequence of an increased CCR5 binding affinity, since in competitive binding assays on CCR5, both 1P7 and CCL5 have similar IC_{50} values (0.74 nM for 1P7, compared to 2.9 nM and 1.56 nM for CCL5 and CCL3, respectively) (Fig 1C). Finally, as expected the native chemokine CCL5 exhibited strong agonist activity on CCR1 and CCR3 while 1P7 behaved as a very weak partial agonist for CCR1 (Fig 1D, upper panel) and induced a weak calcium signal on CCR3 (Fig 1D, lower panel). Taking account of its superagonist activity on CCR5 and its increased CCR5 selectivity with respect to CCL3 and CCL5, we elected to carry out further work on 1P7 to evaluate its efficacy to induce anti-tumor T cell immunity (Fig 2).

3.1. 1P7-Ig DNA enhances the tumor-specific CD8 cellular response.

CCL5 has been previously reported as an adjuvant in DNA vaccination [22-25]. To compare the adjuvant effect of CCL5-Ig and 1P7-Ig, we co-administered chemokine-Ig plasmids with or

without OVA plasmid by intramuscular route in C57BL/6 mice. In C57BL/6 mice, OVA 257-264 (SIINFEKL) is the immunodominant ovalbumin determinant recognized in the context of H-2K^b [39]. We evaluated the effect of chemokine adjuvants on the amplitude of the OVA-specific CD8⁺ T cell response by tetramer H-2K^b/OVA 257-264 staining on peripheral blood cells at day 15, the peak of the cellular immune response [17]. H-2K^b/OVA 257-264-specific CD8⁺ cells were significantly increased following administration of 1P7-Ig DNA ($P < 0.0001$) and CCL5-Ig DNA ($P = 0.0003$) compared to DNA Ig control group, only when co-administrated with OVA DNA (Fig 3A and 3B). More importantly, both the percentage of responder mice and intensity of the antigen-specific CD8 T cell response were significantly increased in the DNA 1P7-Ig group ($1.680 \pm 0.244 \%$, $n = 32$) compared to the wild-type CCL5-Ig group ($0.972 \pm 0.148 \%$, $n = 29$, $P = 0.0187$) or the Ig control group ($0.374 \pm 0.050 \%$, $n = 30$, $P < 0.0001$) with 63 %, 41 % and 7 % of responders, respectively (Fig. 3B). We also observed more OVA-specific T cells in the CCL5-Ig group mice than in the Ig control group mice ($P = 0.003$) (Fig 3B). However, control groups that received Ig, CCL5-Ig or 1P7-Ig plasmids alone showed an absence of OVA-specific CD8 T cells demonstrating an increase of cellular immunity to OVA antigen in the presence of chemokine-Ig. Phenotypic and cytokine analysis have shown that all OVA-specific CD8 T cells generated, displayed a CD44^{high} CD62L^{low} phenotype, a fraction of which also produced IFN-gamma (data not shown). Taken together, these observations suggest that these cells are antigen-specific effector T cells that were primed *in vivo*.

In addition to CCR5, CCL5 binds and activates at least 2 chemokine receptors: CCR1 [26] and CCR3 [27]. Given that CCR5 is the CCL5 receptor primarily expressed on lymphoid cells and that 1P7 shows enhanced selectivity towards CCR5 and less toward CCR3, we tested the 1P7-Ig adjuvant effect in CCR5 KO mice after co-administration of DNA coding for OVA and chemokine-Ig (Fig 3B). Controlled experiments showed that CCR5 KO mice and C57/BL6 mice have similar

capacity to generate OVA-specific CD8 T cell responses after OVA protein + CFA or recombinant OVA-MVA (Modified Ankara Vaccinia virus) (Data not shown). However, the capacity of both CCL5-Ig and 1P7-Ig DNA to amplify OVA-specific CD8 cells was completely lost in CCR5 deficient mice (Fig 3B), underlining the importance of CCR5 activation in the adjuvant effect mediated by CCL5 and its analogues.

We conclude that DNA 1P7-Ig is significantly more efficient at enhancing antigen-specific CD8+ T cell immune responses to a DNA vaccine than wild-type CCL5-Ig.

3.3. Co-vaccination based on OVA and 1P7-Ig confers optimal protection against tumor challenge.

Prior to tumor challenge, we evaluated the *in vivo* cytotoxic activity of induced-T cells after DNA vaccination in mice by adoptive transfer of CFSE labeled target cells pulsed or unpulsed with the OVA peptide SIINFEKL. As depicted in Fig 4, a significant increase in cytotoxic activity was observed in mice that received both OVA DNA and chemokine-Ig DNA compared to control groups. This result demonstrates once more antigen-specific T cell responses after DNA vaccination with CCL5 variants. Even though, DNA vaccination with 1P7 might induce a slightly higher cytotoxic activity, no significant differences were observed between CCL5-Ig and 1P7-Ig co-injected with OVA.

In order to test whether the enhanced frequency of antigen-specific CD8 T cell responses obtained using 1P7-Ig as an adjuvant can contribute significantly to control of tumor growth compared to CCL5-Ig adjuvant, OVA-vaccinated mice were challenged with E.G7-OVA tumor cells and monitored for OVA-specific T cell responses and *in vivo* tumor growth. On day 0, C57BL/6 mice were injected with DNA encoding OVA and CCL5-Ig (n = 23), OVA and 1P7-Ig (n = 24) or OVA and Ig (n = 24). As shown above, we found significantly more OVA-specific CD8 T cells in the DNA 1P7-Ig group than in the Ig group ($1.67 \pm 0.35\%$ and $0.51 \pm 0.09\%$, respectively, $P = 0.0063$,

data not shown) at day 15. On day 30, mice were challenged with either E.G7-OVA tumor cells or EL4. Kinetics of tumor growth and percentage of mice bearing tumors are shown in Fig 5A and 5B. No significant differences were observed in the tumor size between the Ig ($5604 \pm 1901 \text{ mm}^3$) and CCL5-Ig ($4361 \pm 2001 \text{ mm}^3$) DNA vaccinated groups while the mean E.G7-OVA tumor size was strongly reduced in 1P7-Ig group ($1585 \pm 365 \text{ mm}^3$, $P = 0.032$) compared to the Ig control group (Fig 5A). However, a similar size of EL4 tumor was observed in all groups (Fig 5B) demonstrating that tumor evolution is controlled by an antigen-dependant mechanism.

We also depicted in Fig 5C, the percentage of vaccinated mice that did not develop a tumor. A significantly higher number of tumor-free mice was observed in the 1P7-Ig group (85 % tumor free mice, $P = 0.01$) and CCL5-Ig vaccinated mice (77 % tumor-free mice, $P = 0.05$) compared to Ig group (43 % tumor-free mice) (Fig. 5C). Finally, we found a positive correlation between OVA 257-264-specific CD8 T cell counts and tumor size ($P < 0.05$). Significantly fewer OVA-specific cells were evident in tumor-bearing mice ($n = 13$) than in tumor-free mice ($n = 28$) ($0.34 \pm 0.05 \%$ and $1.40 \pm 0.21 \%$ anti-OVA CD8 cells, respectively, $P = 0.004$) (Fig. 5D). This data reinforces the major role of T cells in the control of E.G7- OVA tumor growth.

In conclusion, these results confirm that 1P7, a CCR5 superagonist, acts as an effective DNA-encoded adjuvant, showing enhanced efficacy in terms of the level of induction of a specific CD8 T cell response, which can better control tumor development in mice than CCL5, the native CCR5 agonist.

Discussion

Upon antigenic stimulation, establishment of the adaptive immune responses that determines vaccine efficacy is dependent on efficient T cell priming. In this report we have identified, in 1P7, a new CCL5 mutant that functions as a CCR5 superagonist with an increased CCR5 selectivity compared to native CCL5. When presented on immunoglobulin as a dimer (1P7-Ig) and used in a DNA vaccination strategy, the mutant protein provided a potent adjuvant effect with a strong *in vivo* antigen-specific T cell expansion that was efficient enough to protect against an **antigen-expressing** tumor challenge. Our observation that 1P7 is a selective CCR5 superagonist is in accordance with the complete elimination of the adjuvant effect when 1P7-Ig was used in CCR5-deficient mice.

The level of immunogenicity induced by tumor antigens is usually insufficient to elicit strong and efficient immune responses. To compensate for the low level of T cell proliferation, both exogenous administration of cytokines such as IL-2, IL-7 and IL-15 and multiple injections have been proposed to positively regulate effector T-cell survival and proliferation [40]. Similarly, several chemokines such as GM-CSF, CCL5, CCL4, CCL3 as well as Flt3-ligand and other cytokines have been studied in various DNA vaccination strategies [25, 40-48]. This type of adjuvant strategy is also applicable to DNA vaccination: co-administration of plasmid-encoded immunogens and plasmid-encoded CCL5 resulted in augmentation of antigen-specific T helper cell–proliferative responses and Th1 cytokine production such as IFN-gamma [22, 23, 49]. The effect of these strategies on the enhancement of effector T cell responses remained partial however, and we speculated that efficacy could be improved by using a CCR5 superagonist instead of native CCL5. This turned out to be the case. In our tumor challenge model, **when the vaccine antigen is expressed in the tumor (E.G7-OVA)** a significant reduction in tumor size was apparent in mice

receiving 1P7-Ig DNA, but not in mice receiving CCL5-Ig. **In addition, *in vivo* induced antigen-specific T cells displayed cytotoxic activities.** These results strongly argue in favor of a CTL-mediated protection and against a direct anti-cancer effect of CCL5 as proposed previously by our group [21]. While CCR5 agonists based on CCL5 have been successfully used as anti-HIV agents [34, 37, 50, 51], our study provides the first evidence that CCR5 agonists may also function as effective adjuvants in vaccine strategies.

DNA vaccine delivery systems can mimic the antigenicity of infectious organisms and have become a growing field in vaccine technology. DNA vaccine effectively engages both MHC-class I and MHC-class II pathways allowing induction of CD8 and CD4 T cell immune responses [52]. Even though DNA is considered to be a good vector for T cell priming, it remains insufficient to elicit strong immune responses [53]. To compensate for the low level of T cell proliferation, both exogenous administrations of cytokines such as IL-2, IL-7 and IL-15 and multiple injection, as well as an additional use of “boost” vector [54] have been proposed to positively regulate effector T-cell survival and proliferation [40, 41]. We propose 1P7 to be considered as a potent potential adjuvant in DNA vaccination. **It has to be noted that in our work, single DNA vaccination was sufficient to induce a high level of control of tumor growth, while most DNA vaccination studies necessitate multiple DNA vaccination.** In addition, 1P7 was used under the form of a DNA adjuvant and not as purified protein and thus cannot yet be compared to chemically modified chemokines [37].

How might CCR5 superagonists boost immune responses is still under investigation. Firstly, immature DCs exhibit potent chemotaxis in response to CCL5 [55], hence CCR5 activation may be an effective means by which to recruit antigen-sampling cells to the site of immunization. Secondly, following Ag-induced cell differentiation, Th1 CD4 T cells as well as memory and effector CD8 T cells express CCR5 [56, 57]. According to a recent study [58], CCR5 expression

by T cells would not only be important for maintaining T cell-APC interactions, but CCR5 agonists could also act as a costimulator. We therefore suggest that 1P7 (and possibly APC-bound 1P7) could directly enhance T cell priming and activation in the lymph nodes. Thirdly, a number of other CCR5-expressing cell types, including NKs, monocytes and macrophages may also contribute to the antigen-specific T cell response. CCR5 has also been identified as a receptor that selectively triggers IL-12 production by the CD8⁺ subset of DCs [59]. Finally, CCL5 plays a key role in the migration of effector cells from lymphoid organs to inflammatory sites. In our previous work [21], we injected DNA-CCL5 into installed solid tumor, either EL4 or E.G7-OVA. In both types of tumor, CCL5-Ig DNA injection or CCL5 expression induced tumor reduction suggesting an involvement of both adaptive and innate immunity, consequently CCL5-Ig was proposed for a therapeutic vaccination strategy. In this manuscript, we focused on preventive tumor vaccination and demonstrated that DNA 1P7-Ig + DNA coding for a tumor antigen (OVA) enhanced the frequency of tumor-specific T lymphocytes that were able to control tumor growth, thus demonstrating in this model a major role of 1P7-Ig for a preventive vaccination strategy. Therefore, CCR5 superagonists could modulate upstream as well as downstream events of the anti-tumor response. Further investigation using strategies based on either CCR5 superagonists such as 1P7 or CCR5 antagonists such as P1 [34] may help to further elucidate the role of CCR5 in the regulation of cellular immune responses.

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Figure 1. Signaling activity of 1P7

A. 1P7 is a CCR5 superagonist. Duplicate measurements of CCR5 agonist activity in response to various concentrations of CCL5 (■) and 1P7 (○) were made on Hela-P5L cells. Dose curves were fitted using Prism software (GraphPad). **B.** CCR5 internalization induced by CCL5 and 1P7. Steady-state levels of surface receptor on HEK-CCR5 cells were measured by flow cytometry after incubation with chemokines. Levels are expressed as percentages of that of the control (medium, no chemokine). Bar graphs show the mean $\pm$ SD for two independent experiments performed under identical conditions. **C.** 1P7 has similar CCR5 binding affinity to native CCR5 ligands in a competition binding assay using 125 I radiolabelled CCL3 on CHO-CCR5 cells. Each data point represents the mean $\pm$ s.e.m. of quadruplicate determinations, expressed as a percentage of maximum specific binding (binding in the absence of competitor). **D.** 1P7 shows enhanced receptor selectivity over native CCR5 ligands. Measurements of CCR1 (upper panel) or CCR3 (lower panel) agonist activity at 300 nM of CCL5, CCL3, CCL4 and 1P7 were made on HEK-CCR1 or HEK-CCR3 cells respectively. At this concentration, 1P7 gave a maximum response closed to 5 and 30 % of that of CCL5 for CCR1 and CCR3 respectively

Figure 2. Experimental setting

Mice were immunized intramuscularly with 100 μ g of OVA plasmid + 100 μ g of CCL5-Ig, 1P7-Ig or Ig plasmids at day 0. Each animal was injected subcutaneously in the right flank with $2 \cdot 10^4$ E.G7-OVA tumor cells at day 30.

Figure 3. Monitoring of OVA-specific CD8⁺ T cells following genetic vaccination with OVA and chemokine-Ig

A. Representation of cytofluorometric analysis of fresh peripheral blood mononuclear cells from C57/B6 mice 15 days after co-injection with OVA plasmid and Ig, CCL5-Ig or 1P7-Ig plasmids. The percentages of circulating CD8⁺ T cells stained with the H-2Kb/OVA 257-264 tetramer are indicated for the indicated adjuvant groups **B.** Monitoring of OVA-specific CD8⁺ T cells on day 15 in wild type C57/B6 mice injected with indicated chemokine-Ig plasmids alone (circles, n = 12 in each group) or co-injected with OVA plasmid plus Ig plasmid (open triangles, n = 30), CCL5-Ig plasmid (grey triangles, n = 29) or 1P7-Ig plasmid (black triangles, n = 32). C57/B6 CCR5 KO (CCR5^{-/-}) mice are co-injected as above with OVA plasmid and indicated chemokine-Ig plasmids (squares, n = 7 in each group). Comparisons are made using the Student *t* test.

Figure 4. *In vivo* evaluation of cytotoxic activity after DNA vaccination

Mice were injected as above with either OVA, Ig or chemokine-Ig plasmids alone or co-injected with OVA plasmid and chemokine-Ig plasmids (n = 3 in each group). On day 15 after DNA vaccination, mice were intravenously injected with CFSE labeled target cells pulsed or unpulsed with the OVA peptide SIINFEKL. Cells were recovered 20 h after transfer and analyzed for CFSE intensity in a flow cytometric assay.

Figure 5. Monitoring of tumor-challenged animals

Inoculation of tumor cells was performed 30 days after DNA vaccination. Tumor development in each animal co-immunized with OVA plasmid + Ig, CCL5-Ig or 1P7-Ig plasmids was monitored until day 30 following tumor-challenge. **A.** Each vaccination group was injected with E.G7-OVA cells: Ig group (n=14), CCL5-Ig (n=13) and 1P7-Ig (n=14) (NS: non significant). **B.** Monitoring of EL4 tumor size in animal groups described above (n = 6 in each group). **C.** Monitoring of tumor free mice following E.G7-OVA tumor challenge in mice co-immunized with OVA plasmid plus

Ig, CCL5-Ig or 1P7-Ig plasmids. **D.** Proportion of antigen-specific CD8⁺ T cells in tumor-free (T⁻) or tumor-bearing mice (T⁺). Percentage of OVA 257-264 specific CD8⁺ T cells were quantified on day 15 and tumor size were measured at day 24 after tumor challenge.

Dorgham et al., Figure 1

A **B**

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