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Optimized dosing of a CCR2 antagonist for amplification of vaccine immunity

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Abstract

We have recently discovered that inflammatory monocytes recruited to lymph nodes in response to vaccine-induced inflammation can function as potent negative regulators of both humoral and cell-mediated immune responses to vaccination. Monocyte depletion or migration blockade can significantly amplify both antibody titers and cellular immune responses to vaccination with several different antigens in mouse models. Thus, we hypothesized that the use of small molecule CCR2 inhibitors to block monocyte migration into lymph nodes may represent a broadly effective means of amplifying vaccine immunity. To address this question, the role of CCR2 in monocyte recruitment to vaccine draining lymph nodes was initially explored in CCR2^{-/-} mice. Next, a small molecule antagonist of CCR2 (RS102895) was evaluated in mouse vaccination models. Initial studies revealed that a single intraperitoneal dose of RS102895 failed to effectively block monocyte recruitment following vaccination. Pharmacokinetic analysis of RS102895 revealed a short half-life (approximately 1 h), and suggested that a multi-dose treatment regimen would be more effective. We found that administration of RS102895 every 6 h resulted in consistent plasma levels of 20 ng/ml or greater, which effectively blocked monocyte migration to lymph nodes following vaccination. Moreover, administration of RS102895 with concurrent vaccination markedly enhanced vaccine responses following immunization against the influenza antigen HA1. We concluded that administration of small molecule CCR2 antagonists such as RS102895 in the immediate post-vaccine period could be used as a novel means of significantly enhancing vaccine immunity.

Keywords

Immunization; Chemokine; Monocytes; MCP-1; Immune; Antibody

1. Introduction

Increasingly, attempts to boost vaccine immunity in patients with cancer and chronic infectious diseases have focused not only on improvements in vaccine adjuvants, but also on the negative regulatory role of immune suppressive stromal cells in tumor and lymphoid tissues [1,2]. Macrophages are known to suppress vaccine immunity in patients with cancer,

and recent studies suggest that inflammatory monocytes may play a similar role [3]. Moreover, tissue infiltration with macrophages in individuals with chronic infections with organisms such as *Mycobacterium tuberculosis* and *Trypanosoma brucei* can suppress immune responses [4,5].

However, much less is known about the role of monocytes in the acute regulation of immune responses to vaccination in healthy individuals. Recent studies point to a role for monocytes in regulating early vaccine responses. For example, HIV infected individuals with lower vaccine-induced blood monocyte counts had greater resultant antibody titers compared to those with high monocyte responses to vaccination [6]. In addition, vaccination with the live attenuated BCG vaccine elicited a population of myeloid cells that inhibited T cell responses by suppressing T cell proliferation [4]. We have recently discovered that CCR2+ inflammatory monocytes potently and rapidly downregulate cancer vaccine responses following immunization with non-replicating vaccines in mice by suppressing T cell responses [2]. Importantly, we found that monocyte depletion with liposomal clodronate at the time of immunization could significantly amplify vaccine immunity. Similar amplification of vaccine immunity was also observed following treatment of mice with the CCR2 antagonist drug RS102895. However, in that study dosing of the small molecule CCR2 antagonist drug was not optimized for vaccine enhancement. Thus, there was reason to believe that further improvement in vaccine immunity could be achieved by optimized dosing protocols for use of a CCR2 antagonist as a novel vaccine “adjuvant–adjuvant”.

Monocytes can differentiate into DC or macrophages, depending on recruitment signals and environmental clues. Chemokines regulate the recruitment of monocytes to sites of infection, tissue damage, and ischemia [7,8]. CCL2 (MCP-1) and CCL7 (MCP-3) are the primary chemokines that regulate monocyte recruitment in response to inflammation [9]. Genetic deletion of CCL2 or CCL7 expression (or deletion of the CCL2 receptor, CCR2) results in reduced mobilization of monocytes from the bone marrow into the blood stream and an inability to recruit monocytes into local sites of inflammation [8]. Furthermore, increased serum concentrations of CCL2 are associated with exaggerated monocyte infiltration into tissues and exacerbation of disease in inflammatory conditions such as rheumatoid arthritis [10], atherosclerosis [11], and coronary artery disease [12]. Because of this, specific small molecule CCR2 antagonists have been developed and evaluated in clinical trials for treatment of rheumatoid arthritis [13], type 2 diabetes, and multiple sclerosis [14].

A number of small molecule inhibitors of CCR2 signaling have been developed, including spiropiperidine-containing compounds such as RS102895 [14]. RS102895 was shown to bind specifically and with relatively high affinity to the β subunit of the CCR2 receptor, resulting in potent inhibition of CCR2 signaling [15]. In previous studies, intraperitoneal (i.p.) administration of RS102895 at a dose of 5 mg/kg was shown to reduce monocyte recruitment in mice exposed to inflammatory stimuli [16]. The ability of RS102895 to potently suppress CCR2 signaling and monocyte recruitment suggested that the compound might be useful for blocking the immune suppressive effects of monocytes during early vaccine responses. Indeed, we recently found that RS102895 was effective at enhancing vaccine immunity in mice [2]. However, effective dosing parameters for RS102895 have not been established previously with vaccine immune enhancement and lymph node monocyte recruitment inhibition as pharmacodynamic endpoints.

Therefore, we conducted studies to optimize the use of RS102895 as a novel vaccine immunity amplification agent. A mouse model of vaccination and lymph node monocyte migration was established to provide a direct pharmacodynamic endpoint for RS102895 dosing studies, in addition to augmentation of vaccine immunity. We found that more

frequent dosing of RS102895 to maintain plasma drug levels > 20 ng/ml was associated with significantly greater inhibition of monocyte recruitment to vaccine draining lymph nodes and enhancement of vaccine responses. Based on these results, we provide guidelines for appropriate dosing schedules for use of this drug as a novel agent for broad amplification of vaccine immunity.

2. Methods

2.1. Animals

Mice, C57Bl/6N or ICR, were ordered from Harlan laboratories (Denver, CO). CCR2^{-/-} mice, on C57Bl/6 background, were bred in-house. All mice were housed in an AAALAC accredited facility and all animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee.

2.2. Biochemicals

RS102895 was purchased from Sigma Aldrich (St. Louis MO). The drug was initially dissolved in DMSO at a concentration of 10 mg/ml and then adjusted to final concentrations in distilled water or tissue culture medium. Vaccine adjuvants were prepared using DOTAP-cholesterol liposomes (Sigma-Aldrich) and non-coding plasmid DNA, as described previously [17]. Recombinant HA1 protein was obtained from Immunotech (Foster City, CA). Antibodies for flow cytometry were purchased from eBioscience (San Diego, CA) unless otherwise indicated. Mouse rCCL2 was purchased from Biolegend (San Diego, CA).

2.3. In vitro monocyte migration assays

To procure inflammatory monocytes for in vitro assays, mice were administered 1 ml aged brewer's thioglycolate (BD) by i.p. injection and peritoneal washes were performed 72 h later to collect peritoneal lavage cells. Cells were incubated with RS102895 for 1 h at indicated concentrations and then 3×10^5 cells were added to the top of Boyden chambers with 5 μ m pores (Millicell plates, Millipore, Billerica, MA). Bottom chambers contained medium with or without 30 ng/ml mouse recombinant CCL2 (Biolegend). Cells were allowed to migrate for 6 h at 37 °C. The top of the membrane was then aspirated and carefully cleaned with cotton tipped applicators prior to fixing and staining the cells on the bottom side of the membrane with 0.3% crystal violet in 10% ethanol for 20 min. The membranes were washed with distilled water and mounted on microscope slides (Fisher) for microscopic counting. Five 40 $\times$ fields were randomly selected for counting and the mean number of migrated monocytes per field was determined for each sample.

2.4. In vivo monocyte migration assay

To trigger in vivo monocyte migration, 50 μ l of vaccine with adjuvant was administered in the rear footpad of one leg and the nearest draining lymph node (popliteal) was collected 6–24 h later. The lymph node tissues were processed into single cell suspensions and immunostained for identification of inflammatory monocytes by flow cytometry.

2.5. Flow cytometry

At indicated time points, popliteal lymph nodes draining footpad vaccination sites were harvested and processed into single cell suspensions using cell strainers (BD, Durham, NC). Cells were immunostained with antibodies in FACs buffer (PBS plus 2% FBS and 0.01% sodium azide) directed against the following determinants: CD11b (clone M1/70), Ly6C (clone AL-21) (BD Pharmingen), Ly6G (clone 1A8) (BD pharmingen), and CCR2 (R&D Systems, Minneapolis, MN). Immunostained cells were analyzed using a Cyan ADP flow

cytometer (Beckman-Coulter Brea, CA) and data were analyzed using Summit software (Beckman-Coulter) and FlowJo software (Ashland, OR).

2.6. Mass spectrometry

For pharmacokinetic analysis of RS102895 drug concentrations, mice (n=3/group) were dosed by i.p. injection of 5 mg/kg RS102895 in a volume of 100 μ l water and were euthanized at the indicated time points. Plasma was prepared by centrifugation of blood collected by cardiac venipuncture into heparinized syringes. Control plasma was collected from strain-matched mice of similar age and was spiked with RS102895 to prepare a standard curve of ranging from 0 to 1000 ng/ml. Aliquots of 100 μ l plasma from each sample or standard was spiked with 10 μ l internal standard (a related spiropiperidine compound RS504393 at 300 pg/ml, Santa Cruz) and extracted with 200 μ l acetonitrile containing 0.1% formic acid. Samples were vortexed for 10 min then centrifuged at 14,000 $\times g$ for 10 min and the resulting supernatant was collected.

Supernatants (200 μ l) were transferred to HPLC vials and analyzed by tandem mass spectrometry with the following parameters: positive ion electrospray ionization (ESI) mass spectra were obtained with a MDS Sciex 3200 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems, Inc., Foster City, CA) with a turbo ionspray source interfaced to an Agilent 1200 Series Binary Pump SL HPLC system (Santa Clara, CA). Samples were chromatographed with an XBridge Phenyl, 2.5 μ m, 4.6 $\times$ 50 mm column (Waters Corporation, Milford, MA) protected by a C18 guard cartridge, 4.0 $\times$ 2.0 mm (Phenomenex, Torrance, CA). An LC gradient was employed with mobile phase A consisting of 0.1% formic acid and mobile phase B consisting of acetonitrile with 0.1% formic acid. Chromatographic resolution was achieved by increasing mobile phase B linearly from 30% to 95% from 0.5 to 6 min, maintaining at 95% from 6 to 6.5 min, decreasing linearly from 95% to 30% from 6.5 to 6.7 min, followed by re-equilibration of the column at 30% mobile phase B from 6.7 to 8 min. The LC flow rate was 0.5 mL/min, the sample injection volume was 20 μ L, and the analysis run time was 8 min.

The mass spectrometer settings (for both RS102895 and the internal standard, RS504393, unless otherwise noted) were optimized as follows: turbo ionspray temperature, 350 $^{\circ}$ C; ion spray voltage, 4500 V; declustering potential (DP), 50 V; entrance potential (EP), 8.5 V (RS102895) and 6 V (internal standard); collision energy (CE), 35 V; collision cell entrance potential (CEP), 14 V; collision cell exit potential (CXP), 1.5 V; curtain gas, N₂, (CUR), 15 units; collision gas, N₂, (CAD), medium; nebulizer gas, N₂, 20 units; and auxiliary gas, N₂, 20 units. The predominant product ion was m/z 202.2. Samples were quantified by internal standard reference method in the MRM mode monitoring ion transitions m/z 391.3 $\rightarrow$ 202.2 for RS102895 and m/z 418.3 $\rightarrow$ 215.2 for the internal. The dwell times for each ion transition were 500 ms. Q₁ and Q₃ were both operated in unit resolution mode. Quantitation of RS102895 was based on linear standard curves in spiked blank plasma using the ratio of RS102895 peak area to RS504393 peak area and $1/x^2$ weighting of linear regression. Parameters for the assessment of assay performance were calculated as follows:

$$\text{Accuracy(\%)} = \left(1 - \left| \frac{\text{Theoretical} - \text{Measured}}{\text{Theoretical}} \right| \right) \times 100$$

$$\text{Precision(R.S.D.\%)} = \frac{\text{Standard deviation calculated values}}{\text{Mean calculated values}} \times 100.$$

2.7. Immunization

Mice (n=5 per group) were vaccinated s.c. with 1 µg HA1 in 100 µl cationic liposomal DNA complexes (CLDC) [17] and boosted 10 days later. Mice were treated with RS102895 at both the priming vaccine and the boost vaccine. One group of mice received the HA vaccine alone, while a second group of mice received the HA1 vaccine plus concurrent treatment with RS102895. Mice that were also treated with RS102895 were administered 5 treatments, each at 5 mg/kg administered i.p., beginning at the time of either the prime or the boost vaccination. RS102895 was administered immediately prior to vaccination and again every 6 h thereafter for 5 total treatments. Twelve days after boost vaccination, mice were euthanized. Serum and spleen cells were collected for ex vivo assays.

2.8. Immune assays

Spleen cells were prepared for single cell suspensions using cell strainers. Red blood cells were lysed using ACK solution for 5 min. Spleen cells were plated in 24-well plates at 2×10^6 cells/ml in complete tissue culture medium (RPMI 1640 containing 10% FBS, 1% Pen/strep, and 1% L-glutamine (invitrogen)). For assessment of cell recall responses, cells were restimulated with 50 µg/ml HA1 for 72 h. Supernatants were analyzed for interferon gamma (IFN γ) release using a commercial ELISA (R&D Systems) kit, following manufacturer's instructions.

Humoral immune responses (endpoint titers against HA1) were assessed using anti-HA1 ELISA. Briefly, ELISA plates (Nunc, Rochester NY) were coated overnight with 50 µg HA1 in bicarbonate buffer. During ELISA development, wells were washed 3 $\times$ with PBS containing 0.05% Tween. After blocking with 3% bovine serum albumin for 1 h, serum samples (serial 10-fold dilutions) were allowed to incubate for 2 h, and then washed. Peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was then added for 2 h and developed using tetramethylbenzidine substrate (Sigma Aldrich). The reaction was stopped after 10 min with 1 N HCl and optical densities were determined at 450 nm using a BioTek Synergy HT instrument (Winooski, VT) and analyzed with Microsoft excel software. Positive endpoint titers were determined at 3 standard deviations above OD values for non-immune serum and plotted on a log scale.

2.9. Statistical analyses

All statistical analyses were conducted using Prism5 software (GraphPad, La Jolla, CA). Comparisons between 2 groups were done using a Mann Whitney test. Comparisons between 3 or more groups were conducted using one-way ANOVA with Tukey's posttest. For these analyses, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Vaccination results in rapid migration of inflammatory monocytes to the local draining LN

To assess the kinetics of inflammatory monocyte recruitment to vaccine draining lymph nodes, mice were immunized in the rear footpad and the draining popliteal lymph node was collected at various times from 30 min to 24 h after immunization. These studies utilized a strong vaccine adjuvant, cationic liposomal DNA complexes (CLDC) [17]. Inflammatory monocytes were identified by flow cytometry as CD11b⁺ Ly6C^{hi}. Neutrophils were identified as CD11b⁺ Ly6G⁺. Both cell populations were significantly increased compared to nonvaccinated control nodes within 4 h of vaccination and continued to elevate out to 24 h (Fig. 1A and B).

3.2. Inflammatory monocytes are recruited to vaccine draining LNs via CCR2 mediated chemotaxis

We hypothesized that the chemokine receptor, CCR2, was involved in recruitment of inflammatory monocytes to vaccine draining LNs [18–20]. Previous work by our group [2] has shown that the CCR2 ligand, MCP-1 or CCL2, is upregulated at the site of the inflammatory response as well as the draining LN within 30 min of induction of inflammation. Therefore, myeloid cells in vaccine draining LNs were immunostained with an anti-CCR2 antibody. Analysis of the monocyte population in the vaccine draining lymph nodes revealed that nearly 100% of the monocytes expressed CCR2, consistent with a functionally defined population of inflammatory monocytes [21] (Fig. 2A). Alternatively, only a very low percentage of neutrophils expressed CCR2 (Fig. 2A). To confirm that inflammatory monocytes were being recruited to draining lymph nodes in response to ligation of CCR2, mice lacking CCR2 (CCR2^{-/-}) were vaccinated and lymph nodes were collected 24 h later. Lymph nodes from the opposite, non-vaccinated limb were collected as controls from both wild type and CCR2^{-/-} animals. We observed (Fig. 2b) that vaccine draining lymph nodes from wild type mice contained nearly four times the number of inflammatory monocytes as lymph nodes from CCR2^{-/-} mice. Consistent with the low level of CCR2 expression by neutrophils (Fig. 2A), we observed that CCR2^{-/-} mice still recruited neutrophils to vaccine draining lymph nodes as efficiently as wild type mice (Fig. 2C).

3.3. A single dose of RS102895 is inadequate to block inflammatory monocyte recruitment to vaccine draining lymph nodes

In our previous work, we observed that RS102895 augmented vaccine immunity, but in those studies we did not directly assess the pharmacodynamic effects of RS102895 on inflammatory monocyte migration to lymph nodes over time [2]. Therefore, we next conducted experiments to assess if RS102895 could block vaccine-mediated recruitment of monocytes to the draining LN. To address this question, we first vaccinated mice (n=5 per group) in the rear footpad and concurrently administered a single dose of 5 mg/kg RS102895 intraperitoneally. Twenty four hours later draining lymph nodes were collected. Surprisingly, vaccine draining LNs contained comparable numbers of inflammatory monocytes in untreated and RS102895-treated animals (Fig. 3A). This suggested that RS102895 was not being dosed appropriately in order to control monocyte recruitment under inflammatory conditions and warranted further analysis of the pharmacokinetic profile of this compound.

Previous pharmacokinetic analyses of spiropiperidine-containing compounds have revealed short half-lives [22,23]. Therefore, we hypothesized that the single dose of RS102895 failed to control mobilization of inflammatory monocytes completely during the 24 hour treatment period (Fig. 3A) due to the fact that the drug was not present in the system for long enough to be efficacious. To address this, we conducted pharmacokinetic analysis of RS102895 plasma concentrations in mice over a 24 hour period following a single i.p. administration of 5 mg/kg RS102895. We found (Fig. 3B) that RS102895 had a short half-life (approximately 1 h) and was not detectable in plasma beyond 9 h after administration.

3.4. Administration of RS102895 every 6 h maintained adequate plasma drug concentrations for monocyte migration inhibition

Based on the single-dose pharmacokinetic profile of RS102895 in plasma, it was predicted that dosing every 6 h would be sufficient to maintain adequate drug levels over a 24 h period and therefore sufficient to continuously inhibit monocyte migration. To assess this, a multi-dose treatment study was conducted and trough drug levels were measured in plasma every 6 h immediately prior to administration of the next RS102895 dose. When dosed every 6 h

at 5 mg/kg i.p., trough concentrations of RS102895 did not show accumulation, nor did the drug concentration fall below 20 ng/ml in plasma (Fig. 4A).

Studies were conducted next in vitro to identify the minimal concentration of RS102895 required to block monocyte migration in response to a CCL2 gradient. This assay used thioglycollate-elicited monocyte/macrophages harvested from the peritoneal cavity of mice, as described previously [24]. The ability of the monocytes to migrate towards a CCL2 gradient was assessed using Boyden chambers. We found (Fig. 4B) that RS102895 inhibited monocyte migration in a dose-dependent manner. Moreover, concentrations of RS102895 as low as 20 ng/ml significantly inhibited monocyte migration. Thus, we concluded that the trough plasma concentrations of RS102895 measured during the multi-dose PK study were sufficient to block monocyte migration in response to a CCL2 gradient.

3.5. RS102895 dosed every 6 h blocks inflammatory monocyte recruitment to vaccine draining lymph nodes in vivo

The effects of dosing RS102895 every 6 h at 5 mg/kg on monocyte migration in vivo were assessed. Mice received an initial dose of 5 mg/kg RS102895 immediately prior to immunization in the rear footpad. The mice were subsequently treated with RS102895 every 6 h for a total of 4 treatments, with the final treatment administered 18 h after immunization. Mice were euthanized 12 or 24 h after immunization and inflammatory monocyte and neutrophil recruitment into draining lymph nodes was assessed. Control mice received vaccine only. When dosed every 6 h, RS102895 significantly reduced the number of inflammatory monocytes infiltrating vaccine draining lymph nodes at both 12 and 24 h post immunization (Fig. 5A) whereas single dose RS102895 did not (Fig. 3A). As a control for RS102895 specificity, we also noted that neutrophil recruitment into lymph nodes was unaffected by treatment with RS102895 (Fig. 5B).

3.6. Blockade of CCR2 mediated monocyte recruitment to vaccine draining lymph nodes enhances cell mediated and humoral immunity

To assess the impact of monocyte blockade on vaccine immunity, mice (n=5 per group) were vaccinated against a dominant influenza antigen, HA1, and were simultaneously treated with RS102895 to block monocyte migration. For this study we used the multi-dose treatment regimen that was determined to be most effective at controlling monocyte migration in Section 3.5 (Fig. 5A). Mice received 2 immunizations with 1 µg HA1 using CLDC adjuvant 10 days apart. Mice treated with RS102895 were given i.p. injections of 5 mg/kg immediately before vaccination and every 6 h after for a total of 5 injections, administered during both prime and boost immunizations. Mice were euthanized 12 days after boost and spleen cells were restimulated ex vivo with 50 µg/ml HA1 to monitor T cell cytokine responses to the target antigen. Mice that received RS102895 during immunization (Fig. 6A) had significantly greater IFN γ responses upon restimulation with HA1. Additionally, mice that were vaccinated and treated with RS102895 had significantly higher anti-HA1 IgG antibody titers than mice that received vaccine alone (Fig. 6B).

4. Discussion

In the present study, we show that a small molecule inhibitor of CCR2, when dosed to maintain plasma levels of drug known to be active can effectively enhance vaccine immunity. The surprising potency of RS102895 in augmenting vaccine immunity suggests an important role for inflammatory monocytes in suppressing overall vaccine immune responses, as we have reported recently in a tumor vaccine model [2]. Our findings suggest that RS102895 and related CCR2 inhibitors may, in fact, constitute a new class of vaccine “adjuvant-adjuvants” that can be used to enhance the effectiveness of vaccines in general.

Using a novel *in vivo* monocyte migration assay, we were able to identify plasma concentrations of RS102895 required to block monocyte migration to vaccine draining lymph nodes. Importantly, we found that minimal plasma concentrations of at least 20 ng/ml were required to fully inhibit monocyte migration *in vivo*. However, the CCR2 antagonist was only needed to be administered over a 24 h period (once every 6 h) following vaccination in order to significantly boost vaccine responses, suggesting that a relatively simple drug administration protocol could be used for vaccine enhancement. Since many of the CCR2 small molecule antagonists are orally bioavailable, it is conceivable that the drugs could be taken orally in conjunction with vaccination. Alternatively, it may also be possible to incorporate the CCR2 antagonist drug directly into the vaccine adjuvant formulation.

Our studies revealed that expression of the CCR2 molecule by inflammatory monocytes was essential to lymph node entry, as CCR2^{-/-} mice exhibited a marked reduction in the number of monocytes recruited to vaccine draining lymph nodes (see Fig. 2). However, CCR2^{-/-} mice were still able to recruit neutrophils to vaccine draining nodes (though the numbers of neutrophils recruited were much smaller than the number of monocytes), suggesting an alternative chemokine signaling pathway for neutrophil mobilization following vaccination.

Pharmacokinetic analysis of RS102895 revealed a short half-life (approximately 1 h) following *i.p.* administration. By 9 h after injection, RS102895 was no longer detectable in plasma. Based on single dose PK analysis of RS102895, we determined that dosing every 6 h would prevent complete clearance of the drug over a 24 h period. However, we were unsure if the compound would accumulate in the plasma and perhaps trigger toxicity. Therefore, we conducted multi-dose pharmacokinetic analysis of RS102895, administering 5 mg/kg of drug every 6 h and measuring trough concentrations in plasma over a 24 hour period. With this treatment regimen, trough concentrations remained above 20 ng/ml for the 24-hour period. Importantly, we also determined that a 20 ng/ml concentration of RS102895 was sufficient to significantly block monocyte migration *in vitro* (see Fig. 4). Furthermore, the repeated dosing schedule did not lead to accumulation of RS102895 over the 24 hour period.

The every 6 hour dosing schedule for RS102895 effectively blocked monocyte migration to lymph nodes *in vivo*, an effect that was not seen when a single dose of RS10289 was administered. It also appeared that RS102895 was not having a global effect on reducing cell mobility, since neutrophil migration was unaffected. Utilizing this novel monocyte migration PD endpoint, therefore, can be useful for assessing the effects of specific chemokine receptor antagonists on the intended target cell population, as well as assessing off-target effects on other cell populations.

Importantly, mice were vaccinated against a well-known influenza antigen, HA1, and concurrently treated with RS102895 to block monocyte migration to vaccine draining lymph nodes. RS102895 was administered using the multidosing scheme determined in Figs. 4 and 5 to control monocyte migration *in vitro* and *in vivo*. Those animals that received vaccination with concurrent monocyte migration blockade with RS102895 had a significantly greater IFN γ response when rechallenged with HA1. Furthermore, blockade of monocyte migration during vaccine-induced inflammation enhanced anti-HA1 IgG production by an order of magnitude. Strong cellular and humoral immune responses have been determined to be predictive of influenza protection and clearance, therefore this work suggests that vaccine enhancement by CCR2 blockade could dramatically improve immunity in hard to vaccinate populations [25,26].

In summary, the results of this study suggest that small molecule inhibitors of CCR2 signaling such as RS102895 can be used on a short-term basis to effectively and safely block monocyte migration following vaccination and to significantly amplify vaccine immunity. Future studies may be directed towards identification of more long-acting CCR2 antagonists that can block monocyte migration for periods of >24 h after a single administration. We cannot discount that the monocytes we describe here do not eventually differentiate into other cell types such as macrophages. Additional studies are warranted to further elucidate the terminal differentiation status of these monocytes and what role those cells play in vaccine response. In addition, a more complete understanding of the mechanisms by which inflammatory monocytes suppress vaccine immunity may lead to more specifically targeted treatments to augment immune responses to parenteral and mucosal vaccines.

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Fig. 1. Inflammatory monocyte and neutrophil influx into lymph nodes following vaccination. Mice ($n=5$ per group) were vaccinated in the footpad and vaccine draining popliteal lymph node cells were collected 0.5, 1, 4, 12, or 24 h later and immunostained for analysis by flow cytometry, as described in the Methods section. (Panel A) Populations of inflammatory monocytes ($CD11b^+Ly6c^{hi}$) and (Panel B) neutrophils ($CD11b^+Ly6G^+$) in draining lymph nodes and non-draining lymph nodes were evaluated using flow cytometry. (* indicates statistically significant differences as assessed by one-way ANOVA when vaccinated mice were compared to unvaccinated control animals). This experiment was repeated once with similar results.

Fig. 2.

Monocyte recruitment to vaccine draining nodes is mediated by CCR2-dependent chemotaxis. Panel A. Inflammatory monocytes and neutrophils in vaccine draining lymph nodes were evaluated for CCR2 expression by flow cytometry. Panels B and C. Wild type and CCR2^{-/-} mice were vaccinated in the footpad and vaccine draining popliteal lymph node cells were collected 24 h later. (Panel B) Inflammatory monocytes and (Panel C) neutrophils were quantified using flow cytometry. * indicates statistically significant difference as assessed by one-way ANOVA comparison of vaccinated and control animals. This experiment was repeated once with similar results.

Fig. 3. Single dose RS102895 pharmacodynamic and pharmacokinetic analysis. Panel A. Mice (n=5 per group) were administered a single dose of 5 mg/kg RS102895 i.p. immediately prior to footpad vaccination. After 24 h, cells from draining lymph nodes were collected and inflammatory monocytes were quantified by flow cytometry. Panel B. Pharmacokinetic analysis of RS102895 in plasma.

Fig. 4. RS102895 Multi-dose treatment regimen. Panel A. Mice were administered RS102895 by i.p. injection every 6 h. Plasma concentrations of RS102895 were measured 30 min after the first dose was given and again every 6 h (immediately prior to the next dose) for the next 24 h. Treatments are indicated by arrows and the dotted line represents theoretical drug concentrations between samples based on single dose kinetics determined in 3b. Panel B. Inflammatory monocytes were collected from the peritoneal cavity of thioglycollate stimulated mice and treated with RS102895 at the indicated concentrations in vitro for 1 h prior to adding the cells to Boyden chambers for migration assay. Cells were allowed to migrate towards a 30 ng/ml CCL2 gradient for 6 h before the assay was terminated and

numbers of migrating monocytes determined by manual counting. * indicates statistically significant difference as assessed by one-way ANOVA comparison of RS102895 treated and untreated samples.

Fig. 5. Pharmacodynamic analysis of the effects of multi-dose RS102895 treatment on vaccine-induced cell migration in vivo. RS102895 was administered immediately prior to and every 6 h for a total of 4 treatments after mice were vaccinated in the footpad. Vaccine draining lymph node cells were collected 12 or 24 h later and analyzed by flow cytometry for quantitation of (Panel A) inflammatory monocytes and (Panel B) neutrophils. * indicates statistically significant difference as determined by one-way ANOVA. This experiment was repeated once with similar results.

Fig. 6.

Effects of RS102895 on vaccine immune responses. Mice (n=5 per group) were immunized with 1 μ g HA1 in CLDC adjuvant, and boosted 10 days later, as noted in Methods. One group of animals was treated concurrently with RS102895 administered as 5 consecutive treatments 6 h apart, by i.p. injection. Animals also received a boost vaccine 10 days later (with or without RS102895 treatment). Twelve days after the last immunization, spleen cells and serum were collected for analysis of HA1 immune responses. Spleen cells were restimulated in vitro with HA1 (50 μ g/ml) for 72 h and IFN- γ production assessed (Panel A). Antibody titers to HA1 were determined by ELISA (Panel B). * indicates statistically

significant difference by Mann–Whitney test. Similar results were obtained in one additional experiment.