Introduction

Vaccination with naked DNA holds great promise for number of reasons such as ease of genetic construction, low cost, rapidity of mass production, high stability, and an attractive safety profile [1,2]. However, the immunogenicity of plasmid DNA needs to be improved, and especially in larger animals and humans. Among major contributors to enhanced efficiency are vector design and optimization, and delivery methods such as gene gun [3], electroporation (EP) [4,5], liposomes [6], nano particles [7], and viral capsids [1,2,8]. As concerns the immunogenicity-enhancing effect of EP, employed in the present study, it has been described that changing the field strength [9] or the field orientation (uni- or bipolar) [9] influence the number of transfected muscle fibres and thus the production of protein encoded by the transfected gene. Apart from enhancing transfection efficacy, electroporation may induce inflammatory infiltrates [10,11,12], and enhance production of proinflammatory cytokines [11]; factors that could contribute to potent immunoactivation.

A promising strategy to improve immune responses to protein antigen is to target antigen to antigen-presenting cells (APC). Given their exquisite specificity, antibodies are excellent for this purpose. In pioneering studies, antigens were chemically coupled to antibodies specific for APC; such antigen-antibody conjugates were shown to enhance immune responses [13,14,15]. Later, antigens have been genetically introduced into the C regions of recombinant antibodies engineered to express APC-specific variable regions [16,17,18,19,20,21,22,23]. Antigens have been attached to the carboxy terminal tail of Fab fragments [16] or complete IgG [23], approaches which have certain limitations such as monovalency [16] and bulkiness [23]. In another strategy, 9–37 aa long antigenic peptides corresponding to T cell epitopes substitute loop regions between β-strands in the C-domain [17,18,22,24]. The latter strategy has the advantage that the antibody structure is basically maintained with normal half lives in vivo [20]. The drawback is that the short antigenic peptides often lack conformational determinants and are poor at stimulating B cells and antibody responses. Moreover, the inserted peptides only

DNA Vaccines: MHC II-Targeted Vaccine Protein Produced by Transfected Muscle Fibres Induces a Local Inflammatory Cell Infiltrate in Mice

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Abstract

Vaccination with naked DNA holds great promise but immunogenicity needs to be improved. DNA constructs encoding bivalent proteins that bind antigen-presenting cells (APC) for delivery of antigen have been shown to enhance T and B cell responses and protection in tumour challenge experiments. However, the mechanism for the increased potency remains to be determined. Here we have constructed DNA vaccines that express the fluorescent protein mCherry, a strategy which allowed tracking of vaccine proteins. Transfected muscle fibres in mice were visualized, and their relationship to infiltrating mononuclear cells could be determined. Interestingly, muscle fibers that produced MHC class II-specific dimeric vaccine proteins with mCherry were for weeks surrounded by a localized intense cellular infiltrate composed of CD45+, MHC class II+ and CD11b+ cells. Increasing numbers of eosinophils were observed among the infiltrating cells from day 7 after immunization. The local infiltrate surrounding mCherry+ muscle fibers was dependent on the MHC II-specificity of the vaccine proteins since the control, a non-targeted vaccine protein, failed to induce similar infiltrates. Chemokines measured on day 3 in immunized muscle indicate both a DNA effect and an electroporation effect. No influence of targeting was observed. These results contribute to our understanding for why targeted DNA vaccines have an improved immunogenicity.

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A promising strategy to improve immune responses to protein antigen is to target antigen to antigen-presenting cells (APC). Given their exquisite specificity, antibodies are excellent for this purpose. In pioneering studies, antigens were chemically coupled to antibodies specific for APC; such antigen-antibody conjugates were shown to enhance immune responses [13,14,15]. Later, antigens have been genetically introduced into the C regions of recombinant antibodies engineered to express APC-specific variable regions [16,17,18,19,20,21,22,23]. Antigens have been attached to the carboxy terminal tail of Fab fragments [16] or complete IgG [23], approaches which have certain limitations such as monovalency [16] and bulkiness [23]. In another strategy, 9–37 aa long antigenic peptides corresponding to T cell epitopes substitute loop regions between β-strands in the C-domain [17,18,22,24]. The latter strategy has the advantage that the antibody structure is basically maintained with normal half lives in vivo [20]. The drawback is that the short antigenic peptides often lack conformational determinants and are poor at stimulating B cells and antibody responses. Moreover, the inserted peptides only
fist to certain of the polymorphic MHC molecules in a species, which makes the T cell epitope insertion strategy less attractive as a general vaccine in outbred populations.

To overcome these limitations of previously recombinant antibodies, we generated a novel type of immunoglobulin-based molecules (vaccibodies) that are homodimers, targeting APC by single-chain variable fragment (scFv) specific for mouse MHC class II [25], CD40 [26] and TLR2 [27], or natural ligands like chemokines (MIP-1α and RANTES) [28], and deliver large genetically attached antigens with intact B-cell determinants [25,28] for induction of potent T and B cell responses. Similar to complete antibodies [29], vaccibodies [25] can be delivered by injection of plasmid DNA into skeletal muscle followed by electroporation. Transfected muscle cells synthesize and secrete vaccibodies that can be found in serum and that are absorbed by APC [25]. Thus, antigen-primed APC as well as activation of CD4⁺ T cells can be demonstrated in draining lymph nodes, resulting in protective immunity against tumor challenges [25,28].

However, the events taking place in the muscle with such DNA vaccines that encode APC-targeted proteins have not been studied. To address this issue we have here equipped the MHC II-specific vaccibodies with fluorescent mCherry, which makes the vaccine molecules easy to track. Fibers that produce MHCII-specific vaccibodies become surrounded by infiltrating cells that stain positive for CD45, MHC class II, and CD11b. Large amounts of eosinophils were observed among the infiltrating cells from day 7. Chemokines measured on day 3 after treatment indicates an effect of both DNA-injection and electroporation.

Materials and Methods

Mice

BALB/c mice were bought from Taconic (Ry, Denmark), and were used for experiments between 6 and 10 weeks of age. The study was approved by the Norwegian Animal Research Committee (Oslo, Norway) (Permit number; Id-281 (2005), Id-666 (2007)).

Constructions of mCherry vaccibody and mCherry-containing plasmids

Vaccibodies were constructed as explained by Fredriksen et al. [25]. The gene encoding mCherry [30] was kindly given to us by prof. Tsien (University of California, San Diego, CA). The mCherry gene was amplified from the pRSET-B vector by PCR, using primers introducing either 5’-BsmI, 3’-BamHI, or 5’-SfiI and 3’-SfiI restriction sites. The mCherry gen was cloned either into pLNOH2 between BsmI and BamHI or as antigenic unit in plasmid expressing mCherry-His-Tag sequence for purification of native mCherry protein) was made, using a different 5’-BsmI, 3’-BamHI, or 5’-SfiI and 3’-SfiI restriction sites. The mCherry gen was cloned either into pLNOH2 between BsmI and BamHI or as antigenic unit in the vaccibody format between SfiI sites. In addition, a pLNOH2 plasmid expressing mCherry-His-Tag (for purification of native mCherry protein) was made, using a different 5’-BsmI, 3’-BamHI, or 5’-SfiI and 3’-SfiI restriction sites. The mCherry gen was cloned either into pLNOH2 between BsmI and BamHI or as antigenic unit in the vaccibody format between SfiI sites. In addition, a pLNOH2 plasmid expressing mCherry-His-Tag (for purification of native mCherry protein) was made, using a different 5’-BsmI, 3’-BamHI, or 5’-SfiI and 3’-SfiI restriction sites. The mCherry gen was cloned either into pLNOH2 between BsmI and BamHI or as antigenic unit in the vaccibody format between SfiI sites. In addition, a pLNOH2 plasmid expressing mCherry-His-Tag (for purification of native mCherry protein) was made, using a different 5’-BsmI, 3’-BamHI, or 5’-SfiI and 3’-SfiI restriction sites. The mCherry gen was cloned either into pLNOH2 between BsmI and BamHI or as antigenic unit in the vaccibody format between SfiI sites.

Constructions of mCherry vaccibody and mCherry-containing plasmids

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Quantification of cytokines produced by plasmid-injected muscle

BALB/c mice were injected with plasmids encoding either αMHCII-mCherry or αNIP-mCherry (or PBS as negative control) into each quadriceps, followed by EP. Mice were sacrificed 3 or 21 days later, injected muscles were removed, and pooled for each animal. Muscles were washed in PBS, before adding approx. 1 ml RPMI1640 to a total volume of 1.5 ml, before disruption in an omni mixer. The homogenized solution was centrifuged, and supernatant collected and filtered through a 0.45 μm HT Tuffryn membrane (PALL life science). To avoid repeated freeze-thaw cycles, 50 μL of each sample was aliquotted and kept at −70 °C until analysis. Cytokine and chemokine levels were measured using mouse-specific Bio-Plex 23-plex assay (Biorad laboratories, Hercules, CA, US). Samples were adjusted to 0.5% BSA (Biotest, Dreieich, Germany), as were cytokine standards and background blanks. The assays were carried out according to the manufacturer’s instructions. Measurements and data analysis were performed on Bio-Plex system, powered by xMAP technology by Luminex, operated with Bio-Plex Manager 4.1 software (BioRad Laboratories). The instrument was calibrated with the CAL2 settings (LOW RP1 target value) using Bio-Plex calibration beads (BioRad Laboratories). Samples were analyzed as single samples, whereas standards were analyzed in duplicates.

Statistical analysis

All statistical analysis were carried out using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA, USA). The different groups subjected to cytokine analyses were compared and analysed using the Mann-Whitney test. ELISA data were analysed by using two-tailed t-test. p<0.05 were considered statistically significant.

Results

Construction of mCherry-containing vaccibodies

In order to track the vaccibodies as well as to identify their site of production, DNA encoding the fluorescent protein mCherry was inserted into the vaccibody format. mCherry was chosen because of favourable properties like short maturation time (15 minutes) which allows it to be visualized soon after translation; low pKa (4.5) which make it traceable after endocytosis; and good brightness and resistance to bleaching which are preferable properties in microscopy. In addition, the red shifted emission contributes to less disturbance of tissue auto fluorescence, and its monomeric structure and high tolerance for both N- and C-terminal fusions were attractive features for expression as part of vaccibody [37]. mCherry was genetically introduced into the antigenic unit of vaccibodies specific for either MHC class II (I-E a a) (denoted αMHCII) or the hapten 5-iodo-4-hydroxy-3-nitropheneacetyl (NIP) (denoted αNIP) (Figure 1a). Anti-NIP is anticipated not to recognize any molecules in mice, and was therefore used as non-targeted control.

In vitro characterization of the vaccine molecules

To examine whether the vaccine molecules were secreted, Western blotting and ELISAs were performed on supernatants harvested from transfected HEK293 cells. Under non-reducing conditions, vaccibodies were detected in the culture supernatants of vaccibody-producing HEK293 cells. The vaccibodies were found to comprise of secreted vaccibody molecules with the fluorescent mCherry, which were secreted into the extracellular medium. These results indicate that vaccibodies can be produced in human cells and that they can be secreted into the extracellular medium. These results also suggest that vaccibodies can be used as vaccine candidates in clinical trials.
conditions, bands of approximately 140 kDa were detected while under reducing conditions bands of approximately 70 kDa were observed (Figure 1b). These sizes are consistent with predicted molecular weights and suggest that the mCherry-containing vaccibodies mainly are secreted as dimers. A battery of ELISAs verified that the vaccibodies were secreted from transfected HEK293 (Figure 1c). ELISAs confirmed that the non-targeted control, aNIP-mCherry, bound to NIP-conjugated BSA (Figure 1c). Moreover, the aMHCII-mCherry bound to spleen sections from BALB/c (Figure 1d) but not from C57BL/6 mice (data not shown), consistent with maintenance of I-E MHC II specificity. aNIP-mCherry did not bind spleen sections (Figure 1d). These results demonstrate that the two different targeting units maintained their specificity in the vaccibody format.

The mCherry signal is associated with only a few nuclei in a muscle fiber

We wanted to visualize the vaccine proteins in the muscles after DNA injection and electroporation (EP). In order to increase the control over the electroporation procedure, and to make it easier to localize the transfected muscle fibers, we did sight-guided DNA-injection and EP of muscle soleus, followed by suture. Before analysis by fluorescence microscopy, the mice were anesthetized, and the skin reopened to expose the muscle. Muscle fibers transfected with mCherry vaccibodies emitted fluorescence (Figure 2a). Nuclei were visualized by bathing the muscle during microscopy in Ringer-acetate solution containing DAPI (a fluorescent dye that binds DNA) (Figure 2b).

One muscle fiber/myocyte of muscle soleus, being approximately 7 mm long may contain around 400 nuclei [38].

Figure 1. Characterization of mCherry-containing vaccibodies. (a) Schematic drawing of the vaccibody format [25] which consists of three functional units: a targeting unit, a dimerization unit (hinge h1 and h4 joined to the C3 domain derived from human IgH chain γ3) followed by an antigenic unit. DNA encoding αMHCII or αNIP (non-targeted control) was inserted into the targeting unit, and DNA encoding mCherry or scFv of the myeloma protein M315 (negative control) was inserted into the antigenic unit. (b) The mCherry-containing vaccibodies form dimers. DNA encoding the vaccibodies was transfected into HEK293 cells, and the supernatants were either untreated (-ME) or treated with mercaptoethanol (+ME) prior to SDS-PAGE. Blotted proteins were detected by an anti-mCherry antibody [32]. (c) DNA encoding the vaccibodies or mCherry was transfected into HEK293 cells, and the supernatants were analyzed by ELISA as indicated. (d) aMHCII-mCherry but not aNIP-mCherry bound to sections of spleen from BALB/c. Stippled lines indicate the border between the red pulpa (RP) and the white pulpa (WP).

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Figure 2. Muscle fibers transfected with aNIP-mCherry visualized in vivo in mice. DNA encoding aNIP-mCherry was injected into the soleus muscle followed by EP. One day later, the dermis was opened and mCherry (red) indicative of vaccibody expression was visualized by fluorescence microscopy of live muscle in vivo in anesthetized BALB/c mice. (a) Fluorescence microscopy of longitudinal, mid section of soleus muscle. The muscle belly boundary is indicated by dotted lines. Scale bar, 1 mm. (b) Fluorescence microscopy of two transfected muscle fibers. The dotted lines indicate the boundaries of one muscle fiber. DAPI was added to the muscle surface for staining of the nuclei. Scale bar, 50 μm.

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Inflammatory Cell Recruitment by Targeting to APC

Microscopy of live muscle showed strong mCherry signal juxtaposed to only one or a few nuclei in a long muscle fiber (Figure 2b, day 7 after DNA injection). mCherry signal was observed at the poles of nuclei, probably reflecting the Golgi apparatus, and in round structures within the muscle fiber. In general, there was a gradient of the mCherry signal in the fiber, being highest at the poles of the nuclei and waning with increased distance (Figure 2b).

MHC II-targeted vaccibody enhances antibody responses and recruits immune cells

The vaccibody format was originally designed with the intent to target antigens to APCs, aiming to increase immune responses [25]. To examine whether the MHC II-targeted vaccibodies carrying mCherry in the antigenic unit induced higher levels of anti-mCherry antibodies, DNA encoding either αMHCII- or αNIP-mCherry was injected intramuscularly in quadriceps of BALB/c mice followed by EP. (Note that the electroporation procedure applied to the soleus and quadriceps muscles were different, see Materials and Methods.) Both types of vaccibodies induced antibody responses, however, the kinetics of the responses differed (Figure 3a). Anti-mCherry antibodies were detectable already at day 14 following immunization with αMHCII-mCherry. Antibodies following immunization with αNIP-mCherry appeared later but at day 40 the levels were similar to those induced by αMHCII-mCherry (Figure 3a).

In further experiments, we wanted to examine whether the difference in kinetics was reflected by local reactions in the muscle at the site of DNA injection. Soleus muscles from BALB/c mice immunized with αMHCII-mCherry were harvested after 7 days, cut into sections and stained with an antibody specific for CD45, which is expressed by cells of hematopoietic origin. Microscopy at low magnification revealed relatively few spots with mCherry signal, and an increased number of CD45+ cells present in focal areas throughout the muscle (Figure 3b and 3c, left image). To further identify the localization of the CD45+ cells, sections were immunostained with an antibody to laminin, a glycoprotein found in the basement membrane surrounding muscle fibers. The infiltrating CD45+ cells were localized outside of the basement membrane (Figure 3c, left image). Sections from muscles of BALB/c mice immunized with the non-targeted control (αNIP-mCherry) had comparatively few infiltrating CD45+ cells (Figure 3c, right image). Injection of the αMHCII-mCherry or αNIP-mCherry resulted in approximately the same number of mCherry-expressing muscle fibers (Figure 3c, and data not shown). Thus, these experiments suggest that the αMHCII-mCherry induces a focal inflammatory reaction at the injection site that depends on the targeting specificity.

To study the mCherry signal in more detail, we made longitudinal and cross sections of soleus muscle from mice immunized with MHC class II-targeted mCherry vaccibody (Figure 3d). Similar to the observation in vivo (Figure 2b), strong mCherry signal was found perinuclear to only a few nuclei within the length of a single muscle fiber, indicating a segmented expression of the mCherry vaccibodies within a fiber (Figure 3d). Thus, mCherry observed in a cross section most likely underestimate the number of transfected fibers, because a fiber that appears negative may nevertheless express mCherry at a level above or below the section, as illustrated in an interpretative drawing (Figure 3e). Nevertheless, in further experiments, when characterizing the infiltrates, we chose to investigate the immediate surroundings of fibers that expressed mCherry.

Mononuclear and multilobulated cells are recruited following immunization with MHC class II-targeted vaccibodies

To characterize the CD45+ cellular infiltrate, muscle soleus was harvested 2, 4, and 7 days after immunization and stained with HE. αMHCII-mCherry induced cellular infiltrates present already 2 days after immunization. The infiltrating cells were localized between muscle fibers and the number increased with time (Figure 4a, b). The magnitude of the cellular infiltrate depended on MHC II-targeting since only few cells were observed between the fibers following delivery of αNIP-mCherry (Figure 4b, right panel). The increased cellular infiltration caused by αMHCII-mCherry, compared to αNIP-mCherry was also observed at day 13 (data not shown).

The infiltrate observed after DNA/EP immunization with αMHCII-mCherry, segregated the fibers and disrupted normal muscle morphology (Figure 4b, longitudinal section, left panel). At all time points, the infiltrate consisted of mononuclear cells as well as granulocyte-like cells with multilobulated nuclei (Figure 4a–c). Interestingly, by day 7, numerous cells with morphology similar to eosinophils (multilobulated nuclei and eosinophilic granula) were observed in the tissue (Figure 4c). The eosinophilic infiltrates depended on MHC class II-targeting, since there were no infiltrates of eosinophils following immunization with the αNIP-mCherry (Figure 4b, and data not shown).

Characterization of the cellular infiltrate by immunostaining

αMHCII-mCherry or αNIP-mCherry DNA was delivered to the soleus muscle of BALB/c immediately followed by EP. The muscle was harvested at different time-points after immunization (day 3, 7, and 13, in altogether 8 experiments including a total of 30 mice). Immunostaining of muscles harvested 7 days post immunization, is presented in Figure 5. Similar to what we observed in previous experiments, focal infiltrates were only observed after immunization with the αMHCII-mCherry and not with the αNIP-mCherry (Figure 5). We stained sections for CD11b, a marker of monocytes/macrophages, DCs, neutrophils and natural killer cells. A major fraction of the cells had an intermediate signal for CD11b while a minor fraction of the cells were CD11b bright. In addition, we observed that most of the infiltrating cells were MHC II+ (TIB120).

Cytokines in immunized muscle tissue

To examine whether there was a correlation between cellular infiltration and the cytokine milieu, we measured the levels of cytokines by use of Bio-Plex analyses of homogenized muscles from DNA/EP-immunized BALB/c. Because HE staining revealed an increasing number of infiltrating cells on days 2 and 4 after immunization with the targeted vaccine (Figure 4), we used muscle harvested on day 3 for the cytokine analysis.

Several observations could be made. First, injection of PBS/EP resulted in an increase in 13 out of 23 cytokines, when measured on day 3 compared to day 21 (Figure 6, Table I). Based on the assumption that values obtained on day 21 represent base line levels, our observations indicate that EP and/or the injection volume is a potent method for induction of several pro-inflammatory cytokines. Moreover, when compared to levels obtained by PBS/EP, injection of DNA/EP significantly boosted the expression of 5 out of the 13 cytokines (MCP-1, RANTES, IL-12p40, MIP-1α, MIP-1β). These data suggest that either DNA itself and/or produced vaccibody protein promoted expression of the 5 cytokines. The levels of 3 of the 5 cytokines, IL-12p40, MIP-
Figure 3. Immunization with αMHCII-mCherry followed by EP enhances antibody levels and cellular infiltrates in the muscle. (a) MHC II-targeting promotes antibody responses at early time points. PBS or DNA encoding mCherry or (either αMHCII-mCherry or αNIP-mCherry) was injected into quadriceps of BALB/c mice immediately followed by EP (day 0). Blood samples were harvested at the indicated time points. Serum antibody titers were determined by ELISA employing mCherry as coat. (Each symbol represents mean value of 2 or 3 mice, bars indicates ±SD.) Student’s t-test of endpoint titers induced by αMHCII-mCherry in comparison to αNIP-mCherry gave P-values of 0.01 (IgG) and 0.04 (IgG 2a) at day 14 (asterisk). (b) DNA encoding mCherry-containing vaccibodies was injected into the soleus muscle followed by EP. The muscle was harvested after 7 days, and cross sections of whole muscle were stained by use of anti-CD45 (clone 30-F11). Nuclei were stained by DAPI (blue). Scale bar, 1 mm. (c) DNA was given to BALB/c as described in (b). After 7 days, soleus muscle was harvested and cross sections stained for CD45 (green) and laminin (blue). Red color in the left and right merged micrographs are indicative of αMHCII-mCherry and αNIP-mCherry, respectively. Scale bars, 50 μm. (d) Muscle harvested on day 7 from mice immunized with αMHCII-mCherry. A longitudinal and a cross section is shown in the top and bottom image, respectively. mCherry indicative of αMHCII-mCherry is shown in red and nuclei (DAPI-staining) in blue. White stippled lines indicate a muscle fiber/myocyte that expresses αMHCII-mCherry. Scale bar, 50 μm. (e) Interpretative drawing of a muscle fiber positive for mCherry (red color). The mCherry signal is concentrated around two nuclei (blue), and in puncta in the vicinity. Stippled lines in the drawing denoted a, b, c, and d, represent possible section levels. Some sections will contain both infiltrating cells and mCherry signal (b and d) while in others, only the infiltrating cells will be visible (a and c).

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1α and MIP-1β were enhanced following delivery of DNA, but not following PBS injection, suggesting that electroporation was not sufficient to enhance the levels of these 3 cytokines. Finally, none of the 23 cytokines that were measured showed significant differences after immunization with αMHCII-mCherry or αNIP-mCherry. The results are summarized in Table 1, which also includes a comparison with results published by others.

Discussion

In this study we show that introducing mCherry in the vaccibody format does not appreciably alter the antigenic or fluorescence properties of mCherry, neither does it reduce the specificity of the targeting unit for MHC class II. Furthermore, we show that transfected muscle cells emit mCherry fluorescence already one day post-immunization. Interestingly, by in vivo microscopy on DNA-injected and electroporated (EP) mice, we observed that only one or a few nuclei per transfected muscle fiber produced mCherry. mCherry accumulated in vesicle-like structures in proximity of apparently DNA-transfected nuclei. To what extent the segmented intracellular expression within the muscle fiber is reflected in concentration differences when vaccibodies are secreted to the extracellular fluid is unknown.

It is unclear whether mononuclear cells become transfected after DNA injection and electroporation of muscle [10,39,40]. One study [39] demonstrated that DNA injection into tibialis muscle of mice resulted in DNA uptake both by mononuclear cells and muscle cells. However, only the muscle cells were found to express the transgene, and the expression was enhanced by electroporation. Importantly, the uptake of DNA by mononuclear cells was not influenced by electroporation, and transfected mononuclear cells did not transcribe the transfected gene. Moreover, plasmid DNA was only found in vesicles, indicating endocytosis but not transfection. A later study [40] failed to detect transfected mononuclear cells positive for leucocyte surface markers located in muscle. Moreover, transgene-encoded mRNA was not detected in draining LN. In the most recent study [10], transfected mononuclear cells in the muscle were found to be satellite cells, staining positive for muscle-specific marker desmin, and negative for leucocyte specific surface markers CD3 and CD68. The above mentioned studies are consistent with the present study, since we have yet to find mCherry protein expressed in other cells than muscle fibers themselves, thus, we have no evidence of transfected APC. This observation is consistent with the finding that vaccibodies targeted to MHC-II increased anti-mCherry antibody responses over that seen with non-targeted vaccibody DNA. The targeting effect indicates that vaccibodies secreted by transfected muscle cells bind MHC class II molecules on APC for induction of immune responses. Stated otherwise, if transfection of mononuclear cells had been a major pathway for induction of enhanced immune responses upon DNA/EP immunization, no targeting effect would have been observed.

The electroporation procedure by itself is reported to induce inflammation [10,11], production of chemokines and cytokines...
Figure 6. Cytokines in the muscle following intramuscular injection of DNA and EP. BALB/c were immunized in quadriceps with either PBS or DNA encoding αMHCII-mCherry or αNIP-mCherry, as indicated. After 3 days, muscles from DNA-injected (n = 6/group) and PBS-injected (n = 4/group) were harvested and homogenized before analysis by use of Bio-Plex. Baseline levels, indicated by stippled horizontal lines, represent values measured in muscle isolated 21 days after injection of PBS and electroporation (n = 4). Each symbol represents data from one mouse. Mean values are indicated by horizontal lines. Mann-Whitney test was used to calculate p-values.

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Table 1. Induction of cytokines by electroporation, and further enhancement by injection of DNA.

| Study | EP induced cytokines | DNA enhancement of EP induced cytokines | Enhancement only by DNA/EP in combination |
|-------|----------------------|----------------------------------------|------------------------------------------|
| *This study | IL-2, IL-3, IL-4, IL-5, IL-17, G-CSF, TNF-α, KC, MCP-1, Rantes | MCP-1, Rantes | IL-12p40, MIP-1α, MIP-1β |
| *Peng et al.*[11] | MIP-1α, MIP-1β, MIP-1γ, IP-10, MCP-2, XCL-1 | | |
| *Figarella et al.*[42] | TNF-α, IL-1β | | |

*50 mg of DNA was injected in each quadriceps immediately followed by electroporation (Elgen, Inovio). 3 days later, 23 cytokines were measured in homogenized muscle (Bio-Rad, Bio-plex 23-assay). Cytokines not increased by either EP or DNA/EP were: IL-1β, IL-6, IL-9, IL-10, IL-12p70, IL-13, INF-γ, GM-CSF, and Eotaxin.

*Peng et al.* stimulated tibialis anterior muscle in BALB/c mice with electroporation, and isolated muscle at different time points 2, 4 h and 1, 3d after stimulation. Isolated RNA and measured chemokines with cDNA array.

*Figarella et al.* stimulated gluteus muscle with different combinations of electroporation, non-electroporation, DNA, PBS and empty DNA vector. TNF-α and IL-1β were measured using ELISA on samples isolated at 3 h.
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[11], and influx of a mixture of inflammatory cells [12,40]. In addition, it has been described that injection of DNA, in absence of electroporation, induces upregulation of MCP-1 in muscle, and influx of IFN-γ secreting cells [41]. Although these studies differ in methodology and chemokines/cytokines measured (Table 1), there is an emerging picture that both electroporation and DNA injection can induce chemokine/ cytokine secretion from muscle cells. Our results confirm and extend these previous findings by testing many more chemokines and cytokines. In addition, a novelty of our study is that DNA injection and electroporation appears to act in concert to improve expression of certain cytokines (IL-12p40, MIP-1α, MIP-1β). It may be noted that some cytokines (MIP-1α, RANTES, IL-8, MIG, IP-10, MIP-9α), overlapping with those described herein to be upregulated upon DNA/EP vaccination (Figure 6, Table 1), are also found to be increased in inflammatory diseases in muscle [42].

Interestingly, the different versions of vaccibodies, either αMHCII-mCherry or αNIP-mCherry, did not influence on the expression of cytokines in the muscle. This was surprising since the targeted and non-targeted vaccibodies differed in their abilities to induce antibodies and T cells (present study and [25]). These results indicate that the mechanism for enhanced immunogenicity of αMHCII-mCherry most probably must be sought in the draining lymph node. This suggestion is consistent with previous results demonstrating that αMHCII-vaccibody delivered as DNA/EP induces antigen-priming of APC and proliferation of CD4+ T cells in draining lymph nodes [25] (but not in spleen or non-draining lymph node). Moreover, we have recently found that B cell hybridomas are easily obtained by fusing cells from draining lymph nodes but not spleen of targeted vaccibody immunized mice, indicating that also B cell responses primarily take place in draining lymph nodes [32].

Even though the differences in immunogenicity between αMHCII-mCherry and αNIP-mCherry primarily appears to take place in the draining lymph node, clear cut differences were found in the immunized muscle for the two vaccibody versions. Thus, targeted vaccibodies induced more pronounced local infiltrates of macrophage-like CD45+MHCII+CD11b+cells, neutrophils and late-invading eosinophils. We failed to detect significant numbers of CD19+ B cells and CD11c+ dendritic cells (unpublished results). The latter preliminary observation appears to be at variance with a previous study [12] that described the mixed inflammatory infiltrate induced by DNA/EP to consist of CD19+ B-cells, CD3+CD4+ T-cells, CD11b+ macrophages and CD11c+ dendritic cells. The lack off infiltration of CD11c+ or CD19+ cells in the present study could by a result of methodology of DNA/EP
Inflammatory Cell Recruitment by Targeting to APC

Figure 7. Interpretative drawing of proposed mechanism of action of targeted versus non-targeted DNA vaccines. Injection of DNA (1a,1b) and electroporation (2a,2b) have an adjuvant effect by inducing an inflammatory reaction in muscle resulting in elevated levels of chemokines (3). Transfected muscle cells produce (4, red colour) and secrete the targeted (5, upper) and non-targeted (5, lower) vaccine molecules. By an unknown mechanism, the targeted vaccibody induce an infiltration after >2 days predominantly composed of macrophages/DC, neutrophils and eosinophils (6). Targeted vaccibodies could drain to lymph node either unbound or bound to MHC II* APC, but this remains to be determined. In contrast, non-targeted vaccibodies probably drain to lymph node as unbound molecules. In draining lymph nodes, targeted vaccibodies induce APC-B cell synapses and generation of T helper cells [25], resulting in strong stimulation of antigen specific B cells and generation of antibody secreting plasma cells (7). By contrast non-targeted vaccibodies do not induce APC-B cell synapses, and generation of T helper cells is poor [25] (8), resulting in inefficient plasma cell generation and antibody production.

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Immunization or a real absence. Further studies are clearly warranted to clarify this issue.

We have not been able to define a molecular mechanism for why targeted vaccibodies induced infiltrates, and why the infiltrates appeared to surround the αMHCI-mCherry producing fiber. In particular, we found no differences in levels of 23 cytokines in muscle, related to targeting or non-targeting of vaccibodies (see above). Perhaps subtle differences, unknown factors, or effects masked by the EP-induced inflammation, operate in the vicinity of muscle cells producing αMHCI-mCherry. It is tempting to speculate that ligation of MHC II on a few resident APC in muscle by αMHCI-mCherry, results in unknown chemotactants being produced. However, as stated above, we have so far failed to observe binding of vaccibodies to MHCI* APC in muscle.

Heredia et al. [43] has recently shown that eosinophils infiltrate cardiotoxin-injured muscle in mice and orchestrate, in an IL-4/IL-13 driven fashion, the differentiation of fibro/adipocyte progenitors (satellite cells) into myocytes required for complete regeneration of muscle tissue. In the present study, the late-appearing eosinophilic infiltrate is associated with αMHCI-mCherry. We postulate that this attraction of eosinophils is related to ligation of MHC class II on resident or infiltrating APC. We have failed to observe upregulation of cotaxin or IL-5, but hypothesize that existence a factor “X” could cause influx of eosinophils. It is well known that eosinophils are a major player of innate type 2 protection. It is therefore interesting to notice that the increase in antibodies caused by MHC-II targeting is mainly of the IgG1 subclass, which is known to be associated with Th2 responses. It is tempting to speculate that provision of MHCIi ligation, e.g. by antibodies, can be used therapeutically in muscle diseases to induce infiltration of eosinophils and enhance muscle regeneration.

In summary (Figure 7), the increased immunogenicity of MHCIi targeted vaccine molecules for T cells [25,44] and B cells is probably explained at the level of the draining lymph node. The transfected muscle cells primarily serve as production factories for secreted vaccine proteins. There are two non-exclusive explanations for how the vaccine molecules are transported from muscle to the draining lymph node. One possibility is that the vaccine molecules are transported as soluble molecules with afferent lymphatic vessels and bind to MHC II* APC in the draining lymph node. Another possibility is that the targeted vaccine proteins bind the MHC II* cells that accumulate in the vicinity of the transfected muscle cell. Such vaccibody-primed APC could then migrate to the draining lymph node where they could stimulate T and B cells. We have, however, thus far failed to detect that αMHCIi-mCherry bind to MHC II* infiltrating cells in muscle in vivo. This failure could be due to a too low sensitivity of fluorescence microscopy. Regardless of mechanism of transport, a fraction of the MHCIi-specific vaccine molecules must have maintained intact conformation since stimulation of B cells is enhanced.

We postulate that MHC II-targeted vaccibodies is efficient at stimulating B cells due to formation of APC-B cell synapses in draining lymph nodes, as previously described in vitro [45]. MHCIi-specific vaccine molecules could efficiently bridge APC and B cells. Bivalency of vaccibodies could contribute to synapse formation by increasing avidity for both APC and B cells (Figure 7). As concerns T cell help, MHC II-targeted vaccibody has previously been shown to increase antigen-priming of APC and enhance CD4+ T cell activation [25]. In addition, the B cells could via their BCR accumulate vaccine protein, process it, and present Ag-peptides on their MHCIi molecules to CD4+ T cells (Figure 7). Thus, an APC-vaccibody-B cell synapse should concur with high levels of T cell help. As a consequence, B cells stimulated via their BCR by arrayed vaccine proteins in an APC-B cell synapse, and at the same time receiving strong T cell help, could efficiently develop into antibody secreting plasma cells (Figure 7). The combination of APC-targeting, bivalency of protein, and delivery as DNA, holds promise for establishment of a new generation of subunit vaccines.
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Author Contributions

Conceived and designed the experiments: TOL, JCB, IO KG BB. Performed the experiments: TOL, JCB, IO KG BB. Analyzed the data: TOL, JCB, IO KG BB. Contributed reagents/materials/analysis tools: TOL, JCB, IO KG BB. Wrote the paper: TOL, JCB, IO KG BB.