Induction and analysis of antigen-specific T cell responses in melanoma patients and animal model

Bins, A.D.

Discussion

A short summary of the data described in this thesis

This thesis deals with the problems posed by active tumor immunization in cancer patients. It describes the failure of spontaneous T cell responses to sort a clinical effect in end-stage melanoma patients and studies the effect of a peptide-based vaccine in such patients, detecting no impact on clinical status nor on T cell function. In search for means to improve current active immunization strategies, the intradermal administration of DNA with a tattoo device is explored in mice and macaques, and found to be highly immunogenic in both species.

With regard to "DNA tattooing" it is not realistic to expect this novel immunization method to have a clinical effect in end-stage melanoma patients. In these patients the high tumor load and the tolerized state of the tumor specific T cell repertoire decimate the chances on successful immune mediated tumor eradication. Nevertheless, it may prove beneficial in earlier stages of this disease and in the treatment of virally induced tumors. Additionally, the method may be suitable for immunization against infectious diseases in remote, underdeveloped areas, due to the excellent keeping qualities of DNA and the low-tech application method.

Subsequently, this thesis presents evidence that antigens encoded by DNA vaccines should be stable in order to maximize the efficacy of the vaccine, both upon intradermal and intramuscular injection. Besides this clear message, the finding that antigen stability affects the efficacy of cross presentation contributes to the evolving insight in the mechanisms that govern cross-presentation: it suggests that upon DNA vaccination intact proteins are transferred form the antigen donor cell to the antigen presenting cell.

Finally, this thesis reports on two tools, one that facilitates the monitoring of vaccine induced T cell responses by peptide-MHC tetramers and another to further study the mechanism of dermal DNA vaccines, which may be helpful for their improvement. I'll finalize this thesis by discussing the alleged steps of the mechanism of tattoo DNA vaccination. Doing so, I will try to point out where the bottlenecks may be and make suggestions on how these bottlenecks may be alleviated in order to improve the technology.

Suggestions for the improvement of tattoo DNA vaccination

Transfection efficiency

After DNA tattooing, skin resident cells take up the DNA. In some of these cells the plasmid DNA passes through the cytosol and enters the

nucleus. Once the nuclei of a sufficient number of cells contain a plasmid, the first important bottleneck has been passed; when production starts approximately a few hours after the tattoo, a maximal number of antigen donor cells will help to generate sufficient antigen to induce a T cell response.

To increase the amount of transfected cells after DNA application, the DNA vaccine may be formulated in lipoplexes. This is a routine procedure for *in vitro* transfection. Also, some laboratories have shown that the transfection efficiency upon intradermal DNA administration increases by the addition of certain substances (e.g. aurintricarboxylic acid[41], ATA) to the DNA. An equally simple method leading to the same result is to increase the concentration of the DNA vaccine. However, constraints imposed by the DNA production method limit the maximal concentration. Alternative and technologically more advanced approaches to optimize the transfection efficiency include the addition of a protein group to the DNA, that helps to target the plasmid to the nucleus[42;43].

Asynchronicity of antigen and danger signal: early events

During the DNA tattoo many perforations are made in the dermis. These microscopic wounds will be infected with the skin-resident bacterial flora and injected with the CpG containing DNA vaccine. The danger signals generated this way will lead to the rapid maturation of skin resident APCs and in a later stage to the recruitment and infiltration of immune cells from the circulation.

Upon strong maturation signals, most skin resident APCs migrate away from the skin within a few hours[44;45]. Hence, a substantial part may leave before antigen production starts. Such a 'timing-mismatch' may compromise the efficacy of the method. The preliminary finding that the addition of oligomeric CpG sequences, a very strong adjuvant, to the DNA vaccine abolishes T cell priming fits with this idea.

It should be noted that, before making an effort to compensate this timing issue, more experimental data are required; for example an analysis of tattoo induced APC influx in the DLN, combined with a characterization of the dominant APC type. Furthermore, it goes without saying that this issue only affects cross-presentation mediated T cell priming. Nonetheless, the presence of danger signals before production of the antigen and not during its production, is an unphysiological aspect of tattoo DNA vaccination that needs attention.

It may be difficult to tackle this issue by accelerating the onset of antigen production. Maybe the addition of mRNA encoding the antigen to the DNA vaccine, or the addition of the antigen itself will prove to be beneficial. Although RNA vaccines are known to be functional

intramuscularly , their half life in the dermis is likely to be very short due to the abundant presence of RNAases. Regarding the addition of proteins or peptides it should be noted that 'peptide tattooing' per se does not lead to a T cell response (unpublished data). Nevertheless, mixing RNA or protein to DNA tattoo vaccines may have a synergistic effect. Both additions compromise the easy production of the vaccine though.

It may be more feasible to delay the APC maturation in order to compensate the timing-mismatch. Firstly, simple measures like disinfection of the skin before applying the tattoo may have a positive effect. Secondly, the effect of the elimination of CpG sequences in the DNA vaccine, or of the addition of 'tolerizing'[46] instead of activating CpG sequences, is definitively worth testing.

Asynchronicity of antigen and danger signal: late events

A similar timing issue may come about when, at a later stage, systemic APCs infiltrate the tattooed area. At this time the tattoo induced antigen expression may have waned to suboptimal levels. The current regimen of 3 sequential tattoos may partly compensate this issue. However, in the current regimen each tattoo is placed on a fresh skin patch; hence outside the area where the cellular infiltrate of the previous tattoo (with the recruitment of APCs) is emerging.

If further experiments confirm that the peak of the APC recruitment in the tattooed area occurs after the 'antigen production stop', than one may either accelerate the first or prolong the latter. With regard to prolongation of the antigen expression, exchanging the immediate early cytomegalovirus promoter (IE-CMV) for one that is less liable to silencing (i.e. the EF1-alpha promoter or the CAGGs expression cassette[47;48]) may further prolong antigen expression. On the other hand, the acceleration of APC infiltration may be achieved by the inclusion in the DNA vaccine of genes encoding cytokines that accelerate the recruitment of APCs. The addition of adjuvants is further discussed below. Hopefully, a combination of these measures may eventually lead to a single tattoo regimen.

Partly related to this timing issue, another approach that has been shown to augment the vaccine induced responses is the combination of dermal and intramuscular administration of the DNA vaccine. Intramuscular injection results in expression lasting for months, and preliminary results suggest a synergistic effect with intradermal tattoo administration (unpublished data). The mechanism behind this effect is yet unclear, however.

Quantity and quality of APCs: the impact of adjuvants

In light of the alleged late timing issue, the addition of adjuvants that accelerate the recruitment of APCs may be beneficial. In the past, the addition of adjuvants to protein vaccines[49] and intramuscular DNA vaccines[50] has lead to dramatic improvements in vaccine potency, either by augmenting APC recruitment or by accelerating APC maturation. However, as illustrated by the negative effect of the addition of CpGs to DNA tattoo vaccines, finding the right adjuvant for DNA tattooing is not trivial. So far, the addition of DNA encoding GM-CSF, MIP3a, soluble CD70 multimers, and IL-23 have been tested without clear success (unpublished data). Recently, a strong adjuvant effect of flagellin on intradermal DNA vaccination by gene gun was reported[51]. This adjuvant will probably be equally effective in DNA vaccines administered by tattooing. Therefore, it should be the first on the list of candidate adjuvants scheduled for testing.

Other issues

As described in chapter 5, the stability of the encoded antigen has an important impact on the efficacy of T cell induction (as described in chapter 5). Therefore, future vaccines should encode antigens that are inherently stable or antigens that have been modified to increase their stability.

Currently, we do not yet know the intricacies of the cross presentation process, and it is likely that besides the stability of an antigen other properties of the antigen contribute to the efficiency of the cross presentation process as well. As our insight in the presentation of skin-derived antigens increases, additional modifications to the antigens encoded by tattoo DNA vaccines may lead to further improvement.

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