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HER2/neu DNA vaccination by intradermal gene delivery in a mouse tumor model

Gene gun is superior to jet injector in inducing CTL responses and protective immunity

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DNA vaccines are potential tools for the induction of immune responses against both infectious disease and cancer. The dermal application of DNA vaccines is of particular interest since the epidermal and dermal layers of the skin are characterized by an abundance of antigen-presenting cells (APCs). The aim of our study was to compare tumor protection as obtained by two different methods of intradermal DNA delivery (gene gun and jet injector) in a well-established HER2/neu mouse tumor model. BALB/c mice were immunized twice with a HER2/neu-coding plasmid by gene gun or jet injector. Mice were then subcutaneously challenged with HER2/neu$^+$ syngeneic D2F2/E2 tumor cells. Protection against subsequent challenges with tumor cells as well as humoral and T-cell immune responses induced by the vaccine were monitored. Gene gun immunization was far superior to jet injector both in terms of tumor protection and induction of HER2/neu-specific immune responses. After gene gun immunization, 60% of the mice remained tumor-free until day 140 as compared with 25% after jet injector immunization. Furthermore, gene gun vaccination was able to induce both a strong T_H1-polarized T-cell response with detectable cytotoxic T-lymphocyte (CTL) activity and a humoral immune response against HER2/neu, whereas the jet injector was not. Although the disadvantages that were associated with the use of the jet injector in our model may be overcome with methodological modifications and/or in larger animals, which exhibit a thicker skin and/or subcutaneous muscle tissue, we conclude that gene gun delivery constitutes the method of choice for intradermal DNA delivery in preclinical mouse models and possibly also for the clinical development of DNA-based vaccines.

Introduction

Genetic immunization using naked plasmid DNA is attracting increasing interest in tumor immunology since DNA vaccines combine many desirable attributes, particularly under the aspect of clinical applicability. DNA vaccines (1) code for multiple MHC Class I- and Class II-restricted epitopes that may be presented to both CD4$^+$ and CD8$^+$ T cells; (2) lead to a preferential MHC Class I expression of the antigen; (3) can induce both T-cell and humoral responses; (4) can contain CpG-rich sequences that are highly immunogenic; (5) can be produced at comparably low costs as a "general" vaccine being applicable to most individuals across HLA barriers; and (6) are considered far safer than viral vectors.[1–6] DNA vaccines have been widely used in mouse models, in which they have been shown to successfully induce protective immunity against a subsequent challenge with tumor cells, as well as in transgenic mouse tumor models, which are more likely to reflect the immunological environment of cancer patients.[1,2,4,5,7] However, the immunogenicity of DNA vaccines in humans and large animals has so far been largely disappointing.[8] There is an ongoing debate concerning the main reasons for the failure of genetic vaccines in humans, but the most important topics are related to dosing, application route, tissue distribution, use of adjuvants and differences in Toll-like receptor (TLR) expression patterns between mice and humans.[1–5,9–11] With respect to the application route, the first method described was intramuscular injection, which is able to induce significant T_H1-polarized immune responses in mice.[12] This method was subsequently improved by combining it with electroporation.[4,13–15] During the past two decades, several other application routes have been investigated, most of which basically rely on intramuscular or intradermal gene transfer. In order to avoid a direct intradermal injection by syringe and needle, which is difficult to perform in a reproducible fashion, injection techniques such as gene gun particle bombardment, jet injector immunization and skin tattooing were introduced.[1,2,4,5,16–19] Gene gun delivery and jet injector are particularly attractive methods from a

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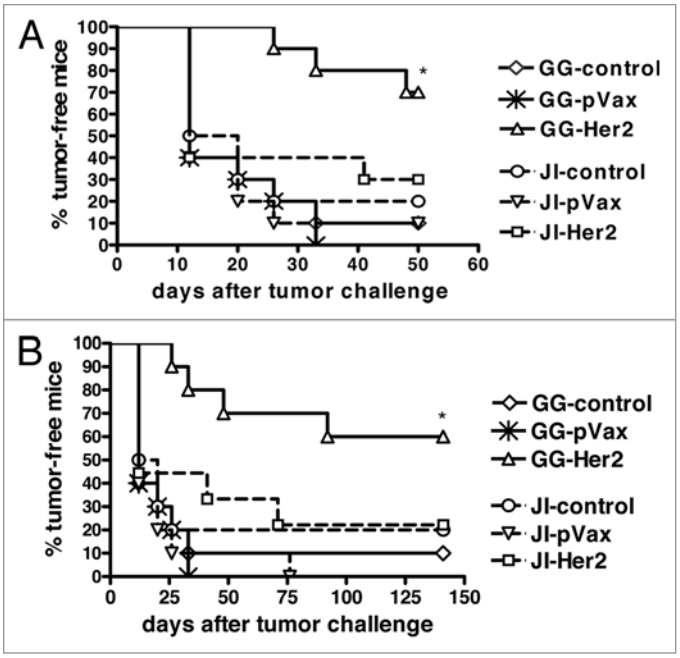

Figure 1. Short- and long-term tumor protection by intradermal DNA vaccination using gene gun or jet injector: percentage of tumor-free mice after vaccination and tumor challenge. **(A and B)** Wild-type (WT) BALB/c mice were immunized by gene gun (GG) or jet injector (JI) delivery with pDNA(HER2/neu), mock vector (pVax) or gold particles/PBS on days 1 and 15. On day 25 tumor challenge was performed with 2×10^5 HER2/neu$^+$ syngeneic D2F2/E2 tumor cells. Tumor growth was then monitored thereafter until day 140. n = 10 for each group of mice. (**A**) Short-term protection (day 50). (**B**) Long-term protection (day 140). * = statistically significant vs. all other groups ($p < 0.05$).

clinical point of view: they apply DNA to the skin avoiding high injection volumes, which can be painful for patients, particularly if repetitive immunizations are being performed. Furthermore, they both primarily target the skin, which is characterized by a high density of different subpopulations of antigen-presenting cells (APCs) including Langerhans cells and dermal dendritic cells (DCs).[20–25] In addition, jet injection has already been used for clinical trials, implying that this device also provides advantages under regulatory aspects.[26–30]

HER2/neu is an oncogene belonging to the HER family of receptor tyrosine kinases and is overexpressed in about 30% of patients with breast cancer.[31] Being expressed on the cell surface, HER2/neu can serve as a target for humoral as well as cellular immune responses. T-cell and humoral immune responses targeting HER2/neu have been successfully induced in mice and humans.[7,32–34] We have previously shown in a BALB/c HER2/neu mouse tumor model that the efficacy of an intramuscular HER2/neu-coding DNA vaccine can be increased by the co-expression of chemokines such as the CCR7-ligands CCL19 (also known as Epstein-Barr virus-induced molecule 1 ligand chemokine, ELC) and CCL21 (also known as secondary lymphoid tissue chemokine, SLC).[35,36] In this study, we asked (with respect to a future clinical use) whether (1) the efficacy of a HER2/neu-targeting DNA vaccine in our experimental system can be improved, in terms of protection and anti-HER2/neu immune responses, by different intradermal application methods and (2) whether the gene gun or the jet injector is the preferable device for the preclinical development of DNA-based vaccines.

Results

Protection after a HER2/neu-targeting DNA vaccination by gene gun or jet injector. The efficacy of the two gene delivery systems (gene gun and jet injector) was evaluated in BALB/c mice that had been immunized on days 1 and 15 with a HER2/neu-coding plasmid, namely pDNA(HER2/neu). Control groups were vaccinated with mock vector (pVax) or gold particles/PBS alone. A tumor challenge with D2F2/E2 syngeneic malignant cells was performed on day 25. Using the gene gun approach, 70% of the mice were protected from tumor growth on day 50 after the tumor challenge, whereas jet injector immunization only led to 30% protection (**Fig. 1A**). With regard to long-term protection from tumor growth (day 140), we found that 60% of mice remained tumor-free in the gene gun group, whereas only 25% of the mice treated with jet injector remained devoid of tumors (**Fig. 1B**). In the jet injector group, there was an initial low level of tumor protection during the first 50 d after tumor challenge. While the difference relative to control groups (pVax- and PBS-receiving mice) was statistically significant until day 41, this difference waned over time, and the jet injector failed to convey statistically significant protection in the long-term (**Fig. 1B**).

T-cell immune responses after HER2/neu-targeting DNA vaccination by gene gun delivery or jet injector. In order to compare HER2/neu-specific T-cell responses after immunization by gene gun or jet injector, animals were vaccinated as described above. Seven days after the second vaccination, HER2/neu-specific MHC Class I-restricted immune responses were analyzed by ELISpot assays, pentamer staining and cytotoxicity assays. After gene gun immunization, a strong T_H1-polarized HER2/neu-specific CD8$^+$ T-cell response was detected in ELISpot assays specific for interferon γ (IFNγ) and interleukin-4 (IL-4), whereas jet injector immunization did not induce any T-cell response over background levels (**Fig. 2**). HER2/neu-specific T cells showed a clear T_H1 polarization and the immune response targeted peptides from the extracellular domain of HER2/neu (**Fig. 2**). Results obtained with ELISpot assays were confirmed by pentamer staining, showing a clearly detectable HER2/neu-p63-specific CD8$^+$ T-cell population after gene gun, but not jet injector, vaccination. Interestingly, HER2/neu-p63-specific T cells could be detected in both the spleen and peripheral blood, although the frequency was comparatively higher in the latter compartement (**Fig. 3**).

Since it is generally believed that tumor protection after vaccination strongly correlates with the presence of CTLs, splenocytes from vaccinated mice were restimulated in vitro with the HER2/neu-p63 peptide for 5 d, followed by a standard ^{51}Cr release-based cytotoxicity assay, aimed at detecting CTL activity and comparing the induction of CTLs by the two vaccination techniques. Target cells were either HER2/neu$^+$ D2F2/E2 tumor cells (with D2F2 HER2/neu$^-$ tumor cells as a control; **Figure 4A and C**) or HER2/neu$^-$ D2F2 tumor cells pulsed with the HER2/neu-p63 peptide (**Fig. 4B and C**). This experimental setting was

selected to determine whether CTL de facto recognize tumor cells in a HER2/neu-specific manner and whether HER2/neu-p63-specific T cells (which had been detected by pentamer staining) indeed exert cytolytic activity. Our results clearly show that only the gene gun immunization protocol is able to induce significant levels of HER2/neu-specific CTLs that are able to lyse D2F2/E2 tumor cells in a strictly antigen-specific manner. Indeed, significant levels of HER2/neu-directed CTL activity over background could not be detected after jet injector immunization (**Fig. 4A**). Furthermore, our findings indicate that the HER2/neu-p63 peptide is a critical epitope for tumor rejection in our experimental system.

Humoral immune responses after HER2/neu-targeting DNA vaccination by gene gun delivery or jet injector. The induction of HER2/neu-specific antibody responses by the two different vaccination methods was evaluated using a cytofluorometric assay as described above. The analysis was performed on serum obtained 7 d after the second vaccination. In line with the results of HER2/neu-specific T cell analyses, significant anti-HER2/neu antibody responses (total IgG) were detected only upon gene gun immunization, while the jet injector delivery was unable to induce humoral immune responses over background levels (**Fig. 5A**). Further analysis of the immunoglobulin isotypes showed that humoral immune responses predominantly consisted of anti-HER2/neu antibodies with an IgG1 and—to a lesser extent—IgG2a and IgG2b isotype (**Fig. 5B–D**).

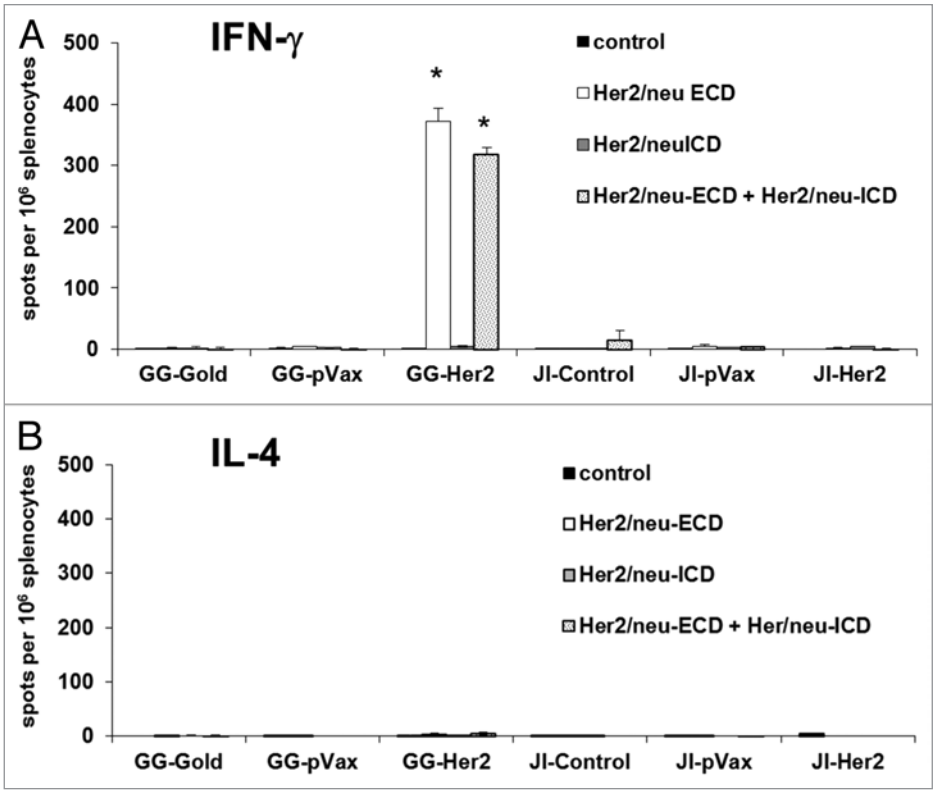

Figure 2. Anti-HER2/neu T-cell immune responses after vaccination. **(A and B)** Splenocytes from wild-type (WT) BALB/c mice intradermally immunized with pDNA(HER2/neu) using gene gun (GG) or jet injector (JI) were stimulated with different peptide combinations (derived from the extracellular domain or the intracellular domain of HER2/neu, or both). Specific T-cell responses were analyzed by a interferon γ (IFNγ)-specific (**A**) or interleukin-4 (IL-4)-specific ELISpot assays. Mice had been immunized with DNA on days 1 and 15. ELISpot assays were performed 7 d after the last vaccination. For ELISpot assays, splenocytes within the different groups of mice were pooled. ECD, extracellular domain; ICD, intracellular domain. * = statistically significant vs. all control groups ($p < 0.05$).

Discussion

Even though the intramuscular delivery of DNA vaccines in combination with electroporation is efficient in mice and allows to reduce injection volumes,[37] dermal application systems still appear to have potential advantages with regard to clinical use in patients. Hence, a comparison of common dermal application systems such as the gene gun and the jet injector, as performed in this study, addressed a clinically important question. The HER2/neu mouse tumor model that we employed was chosen because it is well established, important with respect to a potential clinical use in breast cancer patients and suitable for a detailed analysis of both MHC-restricted T-cell and humoral immune responses.[18,36,38] Our study clearly shows that gene gun vaccination is superior to jet injector immunization in conveying protection against a subsequent tumor challenge and in inducing humoral and T-cell immune responses against HER2/neu. Of note, gene gun DNA delivery—in contrast to jet injection—was able to elicit a very strong CTL response, which is believed to be crucial for tumor rejection.[4,5,7,39,40] However, in order to determine whether gene gun immunization is able to induce long-term immunity against HER2/neu in our experimental system, an additional tumor challenge at a later time point will have to be performed in protected animals. Our results are in line with a previous DNA vaccination study by Trimble, et al. based on the targeting of a viral antigen in mice.[41] These authors report that gene gun immunization leads to better tumor protection and a higher number of antigen-specific $CD8^+$ T cells as compared with jet injector and intramuscular application. In the literature, jet injector systems have been used for gene transfer experiments in different tissues such as muscle, skin, mucosa and tumor lesions.[17,18,26,27,42–47] In addition to many non-immunological strategies, successful DNA vaccination by jet injector has been described in animals including mice, rabbits, cattle and monkeys, as well as in humans,[17,46,48] although these experiments were mostly performed with antigens from infectious agents, which are presumably more immunogenic. Schramm-Baxter, et al. demonstrated that the penetration and dispersion of liquid jets

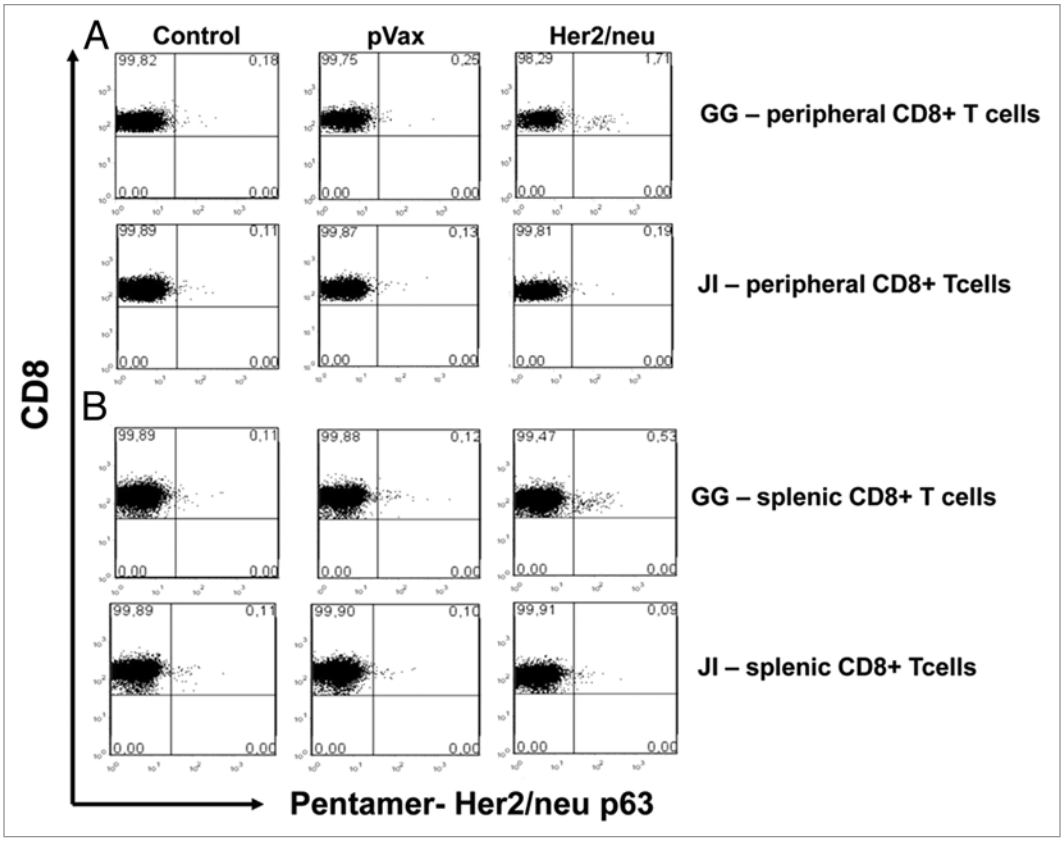

Figure 3. Anti-HER2/neu T-cell immune response after vaccination as detected by HER2/neu-p63-specific pentamer staining. Splenocytes from wild-type (WT) BALB/c mice intradermally immunized with pDNA(HER2/neu) using gene gun (GG) or jet injector (JI) were stained with HER2/neu-p63-specific pentamers. For cytofluoromtetric analyses, splenocytes from mice belonging to the same experimental group were pooled. Splenocytes were analyzed within a $CD3^+$-restricted gate.

in the skin is extremely dependent on the jet power.[49] Therefore, it seems likely that the immunogenicity of jet injector-delivered DNA might be optimized by technical modifications of injection depth, pressure and volume (reviewed in ref. 47). However, even if this would be the case and would lead to better immunogenicity, our data argue in favor of gene gun delivery as the method of choice for intradermal DNA application in mice, since particle bombardement to the skin appears to be sufficiently standardized and is usually confined to the upper layers of the skin, where APCs can be found in abundance.[20–25]

A detailed analysis of the mechanism underlying the different immunogenicity of gene gun and jet injector delivery in our experimental system was far beyond the scope of this study. This said, a closer look at the biological mechanism of gene transfer in gene gun and jet injector delivery might help explain why gene gun is more efficient in inducing HER2/neu-specific immunity. Initially, it was believed that epidermal Langerhans cells (LCs) play a major role in the immune response upon intradermal immunization. However, the basic mechsnisms of immune responses following intradermal immunization have previously been studied in detail. Transfection experiments using cell-specific promoters demonstrated that LCs are in fact completely dispensable for gene gun immunization.[50] Conversely, dermal DCs, which are located in the subepidermal layer of the skin, and non-immune cells such as keratinocytes seem to be the most important drivers of immune responses after gene gun immunization.[51] The authors of this study also concluded that cross-priming (transfected keratinocytes express antigens that are taken up by APCs) is much more important than direct priming by APCs that have been directly transfected.[51] Gene gun particle bombardment primarily targets cells in the epidermal and dermal layers of the skin,[20–22] whereas jet injection delivers a liquid formulation of DNA millimeters to centimeters below the skin surface.[14] This might be particularly disadvantageous in animals exhibiting a thin skin layer and/or a lack of substantial subcutaneous muscle tissue in the area of vaccination. Previous investigations show that transgene expression levels in the skin after jet injection do not predict the magnitude of immune responses,[48] suggesting that cells in deeper layers and/or cells migrating from the skin to regional lymph nodes may play a prominent role in determining the efficacy of the vaccine. Furthermore, gene gun may lead to a stronger local inflammatory response, owing to the huge number of gold particles, causing local injury with a scattered distribution pattern. In contrast, jet injector leads to a more compact distribution of the DNA-containing liquid and—depending on the pressure—can reach the subdermal or even the muscular tissue, which contain a completely different pattern of APCs. In fact, during vaccination, the induction of an appropriate

immunostimulatory microenvironment by cytokines decides whether the immunological outcome is immunity or tolerance. While tolerance results from antigen-presentation in a "non-immunogenic" context (= lack of danger/inflammatory signals), vaccination may elicit strong anti-tumor immune responses, if the vaccine and its adjuvant are able to induce the maturation/activation of APCs and hence the production of T_H1-polarizing cytokines. Furthermore, evidence accumulated during the past years points to a complex interplay between the innate and the adaptive immune system that may determine the final outcome of immunization.[52–56] In this regard, intradermal immunization by gene gun seems to be more immunogenic than jet injection, at least in mouse models such as the HER2/neu$^+$ tumor model used in our study. Curcio, et al. have recently shown that the rejection of a HER2/neu$^+$ mouse tumor upon gene gun vaccination is indeed dependent on multiple, non-redundant innate and adaptive immune mechanisms.[57] Additionally, it has been demonstrated in a mouse vaccination model that gene gun immunization against a viral antigen is able to mimic natural infection, in particular with regard to T-cell responses induced by the vaccine.[58] Although the results of our study are clearly in favor of gene gun delivery, the use of the jet injector technology under strictly predefined conditions may be more appropriate in larger animals (or humans) exhibiting a relatively thick skin layer. Irrespective of this issue, our data indicate that gene gun is the preferable method for delivering DNA vaccines in preclinical mouse models and possibly for future clinical development in a situation of minimal residual disease after surgery or systemic therapy.

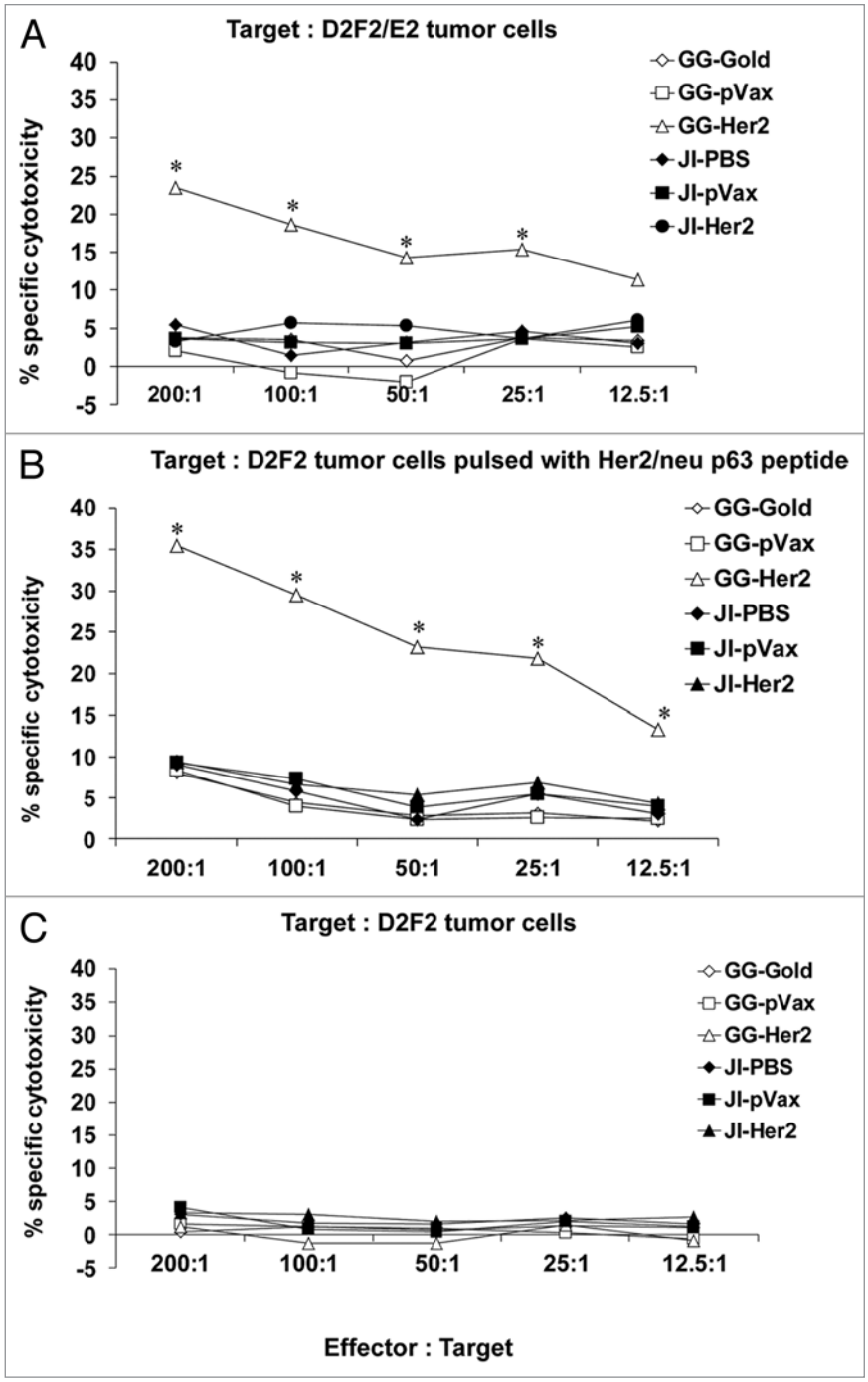

Figure 4. CTL assays after vaccination with gene gun or jet injector. **(A–C)** Wild-type (WT) BALB/c mice were immunized by gene gun (GG) or jet injector immunization (JI) with pDNA(HER2/neu), mock vector (pVax) or gold particles/PBS on days 1 and 15. On day 22, splenocytes were restimulated in vitro for 5 d with irradiated BALB/c 3T3 cells that were pulsed with a HER2/neu-p63 peptide. CTL activity was measured in a standard ^{51}Cr release assay using HER2/neu$^+$ D2F2/E2 tumor cells (**A**), HER2/neu$^-$ D2F2 tumor cells, pulsed with the HER2/neu-p63 peptide (**B**), or HER2/neu$^-$ D2F2 tumor cells only (negative control) (**C**). * statistically significant vs. all other groups ($p < 0.05$).

Materials and Methods

Cell lines. The mouse mammary tumor (MMT) cell line D2F2 is derived from a spontaneous mammary tumor that arose in a BALB/c background from the hyperplastic alveolar nodule (HAN) cell line D2. D2F2/E2 was transfected with the human HER2/neu expression vector pCMV/E2 and the selectable plasmid DNA pRSV/neo. The HER2/neu-expressing cell line D2F2/E2 was maintained in DMEM medium containing 800 μg/mL G418 (Life Technologies). These cell lines were kindly provided by Dr. Thomas Kammertöns (MDC). The human HER2/neu-overexpressing breast cancer cell line SK-Br3 was purchased from The American Type Culture Collection cell bank (ATCC) and used for cytofluorometric detection of HER2/neu-specific antibodies in animal sera. This cell line was maintained in RPMI

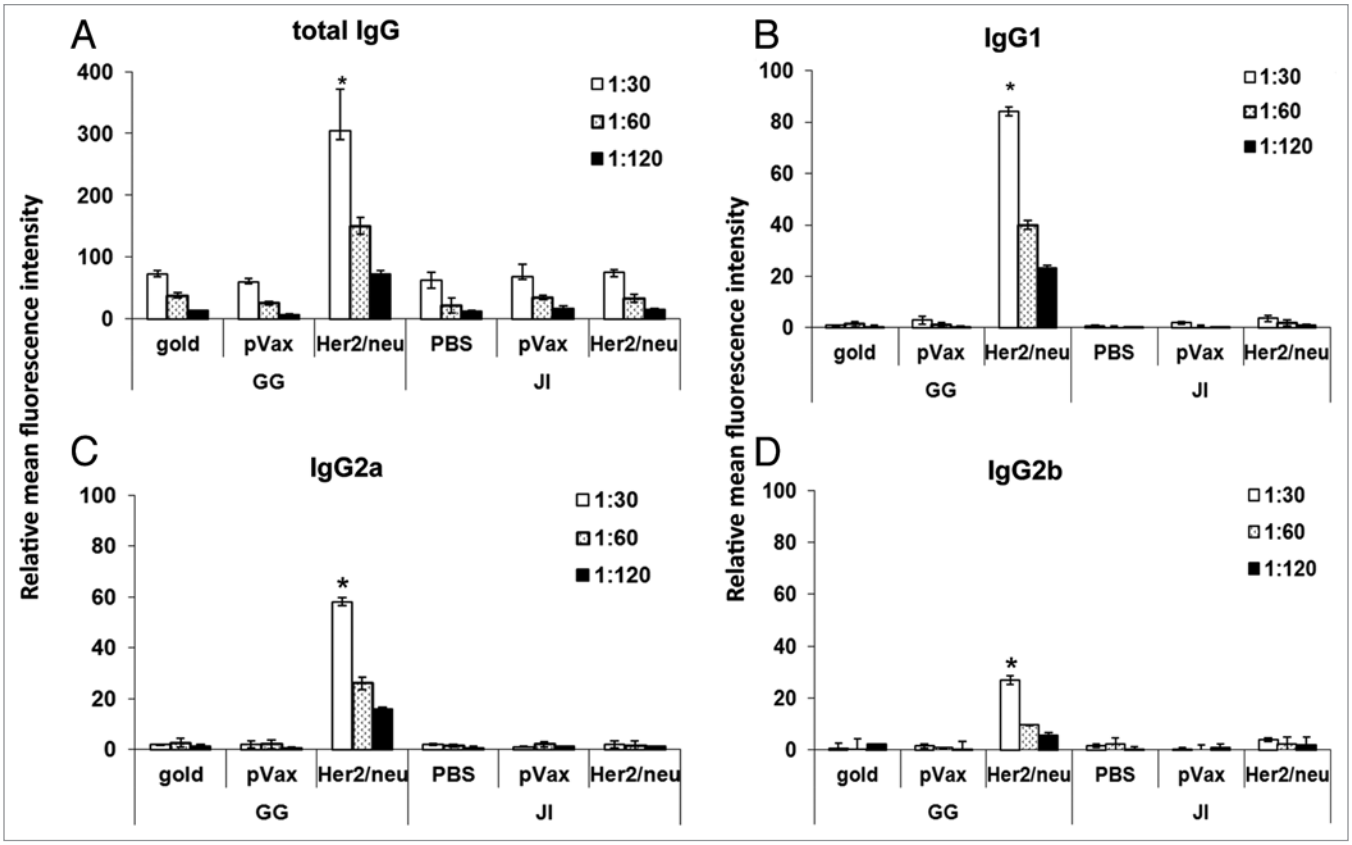

Figure 5. Humoral anti-HER2/neu immune responses after vaccination. **(A–D)** Antibody responses (total IgG and IgG isotypes) against HER2/neu were determined by a cytofluorometric assay in wild-type (WT) BALB/c mice intradermally immunized with pDNA(HER2/neu) using gene gun (GG) or jet injector (JI). Mice had been immunized with DNA on days 1 and 15. Cytofluorometric assays were performed 7 d after the last vaccination. (**A**) Anti-HER2/neu, total IgG. (**B**) Anti-HER2/neu, IgG1. (**C**) Anti-HER2/neu, IgG2a. (**D**) Anti-HER2/neu, IgG2b. * = statistically significant vs. all other groups ($p < 0.05$).

medium (Lonza) supplemented with 10% FCS (Biochrom), 2.5 mM 2-mecaptoethanol (Invitrogen), 0.5 mM sodium pyruvate (Lonza), 2 mM L-glutamate (Lonza), 0.1 mM MEM nonessential amino acids (Lonza) and 100 U/mL penicillin/streptomycin (Lonza) (complete medium, CM). The expression of HER2/neu protein in SK-Br3 cells was confirmed by FACS staining using PE-conjugated anti-human HER2/neu antibody (BD PharMingen). The percentage of HER2/neu expression in the D2F2/E2 cells used in our experiments always exceeded 95%.

HER2/neu-coding plasmid. pVax was purchased from Invitrogen. pDNA (HER2/neu) encoding human HER2/neu was kindly provided by Dr. T. Kammertöns (MDC). The HER2/neu plasmid vector pVax/E2A contains the E2A gene from pCMV/E2A which was cloned into the expression vector pVax. E2A contains mutations at nucleotide positions 2257–2258 that result in a lysine to alanine exchange at position 753 of the amino acid sequence. This alteration leads to elimination of tyrosine signaling in the HER2/neu proto-oncogene, an important safety feature for potential clinical vaccine development. The plasmid was amplified in the *Escherichia coli* X1-blue strain (Agilent Technologies) and purified using the EndoFree Giga-Prep-Kit (Qiagen) according to the manufacturer's instructions.

Animals. Female 6–8 weeks old BALB/c mice (H-2k^d) were purchased from Charles River, and were housed in our animal facility (MDC) under standard pathogen-free conditions. Experiments have been approved by local authorities (LAGeSo) and performed according to the German animal protection law.

Immunization and tumor challenge. Mice were injected twice on days 1 and 15, either by DNA-coated gold particle bombardment onto the shaved abdomen using a Helios gene delivery system (Biorad) or by jet injector (EMS Medical SA) using DNA containing solution of 1μg DNA/μL PBS. For gene gun vaccination, DNA was coated onto 0.8–1.5 μm gold particles following a protocol developed for the helium-driven gene delivery system from Bio-Rad. Two μg DNA per immunization were delivered in two shots with a helium discharge pressure of 300–400 psi. Jet injector immunization was performed by applying five intradermal jet-injections of 10 μL solution per injection, each of which delivered 50 μg DNA in total. Technically, this kind of jet injection-based DNA delivery should be performed with a DNA concentration of 1 μg/μL and allows for a minimum injection volume of 10 μL. This explains the amount of DNA administered with our jet-injection device and is in line with previous studies.[41] Each experimental group consisted of 5–10 mice. Mice were injected with pDNA(HER2/neu) or mock vector (pVax). As further negative controls, uncoated gold particles were used for gene gun immunization and PBS for jet injector vaccination. Ten days after the second vaccination, each mouse was challenged with 2×10^5 D2F2/

E2 tumor cells. The appearance and growth of tumors in the mice were then monitored 1–2 times per week. Progressively growing masses over 1 mm in diameter were regarded as tumors and tumor volumes were calculated as $1/6\ \pi\ d^3$ (d = diameter).

Preparation of splenocytes. Spleens were aseptically removed and single cell suspensions were generated in complete medium. Erythrocytes were lysed using conventional erythrocyte lysis buffer ($EDTA+NH_4Cl+Na_2CO_3$). Finally, splenocytes were washed twice in RPMI 1640 medium and subsequently used for immunological assays.

ELISpot assays. For ELISpot assays, splenocytes were seeded into 4–6 wells (10^6 splenocytes/well) of interferon γ (IFNγ) or interleukin-4 (IL-4) ELISpot plates (ELISpot Kit, PharMingen). Peptides were added at a concentration of 1 μg/mL. Plates were incubated overnight, developed according to the manufacturer's instructions and analyzed using an ImmunoSpot reader system (CTL Europe). Peptide-specific responses were defined as having (1) a ratio of specific peptide:control ≥ 2, and (2) an absolute number of spots > 20. Results were expressed as "spots per 10^6 splenocytes." The following HER2/neu peptides were used: (1) peptides derived from the extracellular domain (HER2/neu-ECD): a: HER2p63–71, TYL PTN ASL; b: HER2p342–350, CYG LGM EHL; c: HER2p369–377, KIF GSL AFL; d: HER2p440–448, AYS LTL QGL; (2) peptides derived from the intracellular domain (HER2:neu-ICD), a: HER2p773–782, VMA GVG SPY V; b: HER2p780–788, PYV SRL LG; c: HER2–2p883–899 KVP IKW MAL ESI LRR RF; d: HER2p907–915, SYG VTV WEL. H-$2k^d$ restriction and potential immunogenicity in mice have previously been shown for most of these peptides.[59] Using the BIMAS epitope prediction algorithm (www.bimas.cet.nih.gov), most peptides were found to be high affinity binders for H-$2k^d$. Only peptides 1c, 2a and 2c were predicted to have a low affinity for H-$2k^d$. All peptides were purchased from Wita GmbH and had a purity of ≥ 95%.

Pentamer staining. To detect peripheral or splenic HER2/neu-p63-specific $CD8^+$ T cells, lymphocytes isolated from the blood of mice on day 6 or from spleens on day 7 after the second vaccination were stained with a PE-labeled pentamer that recognizes the HER2/neu-p63 epitope (ProImmune Ltd.) using the manufacturer's protocol. Lymphocytes were then further stained with a mix of the antibodies containing CD3-FITC, CD8-APC (PharMingen) and CD19 PerCP-Cy5.5 (Biolegend). Stained cells were detected using a BD FACS-Canto cytofluorometer and analysis was performed with the FlowJo software.

Cytotoxicity assays. Splenocytes were restimulated in vitro for 5 d in complete medium in the presence of 10 U/mL IL-2 (Roche) and syngeneic BALB/c 3T3 cells pulsed with 10 μg/mL HER2/neu-p63 peptide (WITA GmbH) as described above. The ratio of splenocytes:tumor cells used for restimulation was 10:1. Peptide-pulsed BALB/c 3T3 cells were maintained for 1 h at 37°C in complete medium and PBS at a ratio of 1:1 and subsequently irradiated with 100 Gy. D2F2/E2 tumor cells ($HER2/neu^+$) or HER2/neu-peptide loaded D2F2 cells were used as target cells in a standard ^{51}Cr release assay. Effector cells and target cells were incubated at different effector:target ratios (12.5:1 to 200:1) for 4.5 h at 37°C, after which 50 μL of culture supernatant was transferred to lumina plates (Packard Bio Science) and radioactivity measured using a scintillation counter (Top Count). The percent specific cytotoxicity was calculated as follows: (% specific cytotoxicity = 100 × (experimental release-spontaneous release)/maximum release-spontaneous release).

Detection of anti-HER2/neu antibodies. Detection of antigen-specific antibodies in the serum by means of flow cytometry offers the advantage that it can be performed even if the respective recombinant protein is not easily available or if its production is very cost-intensive. The assay uses target cells that express the cognate antigen to bind serum antibodies that are detected in a second step by flow cytometry using labeled anti-Ig antibodies.[60] Sera were collected from mice and stored at –20°C until use. Thawed sera were diluted in PBS to 1:30, 1:60 and 1:120 and incubated with 3–5 × 10^5 SK-BR-3 cells for 30–60 min at 4°C. Cells were then washed twice with PBS and resuspended in PBS containing 50 μL biotinylated anti mouse IgG, anti mouse IgG1, anti mouse IgG2a, anti mouse IgG2b or control antibody diluted 1:50 (Perkin-Elmer). Cells were incubated for 30–60 min at 4°C, washed twice with PBS and resuspended in PBS containing 50 μL of streptavidin-APC (BD PharMingen) diluted 1:200. Finally, cells were incubated for 15–30 min at 4°C, washed with PBS and resuspended in FACS-buffer (PBS containing 0.5% BSA, 2 mM EDTA, 0.05% NaN_3) for cytofluorometric analysis. Relative concentrations of anti-HER2/neu total serum IgG or IgG isotypes were calculated by comparing the mean channel fluorescence (MCF) in the different samples. MCF reflects the binding of anti-HER2/neu antibodies to SK-BR3 cells and is therefore a useful measure of humoral anti-HER2/neu immune responses.

Statistical methods. Analysis of variance (ANOVA) of the means of three or more groups was performed using the PRISM software. The Student's t-test was used to compare the means of two groups. p values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization. Annu Rev Immunol 2000; 18:927-74; PMID:10837079; http://dx.doi.org/10.1146/annurev.immunol.18.1.927.
2. Choo AY, Choo DK, Kim JJ, Weiner DB. DNA vaccination in immunotherapy of cancer. Cancer Treat Res 2005; 123:137-56; PMID:16211869; http://dx.doi.org/10.1007/0-387-27545-2_6.
3. Kutzler MA, Weiner DB. Developing DNA vaccines that call to dendritic cells. J Clin Invest 2004; 114:1241-4; PMID:15520855.
4. Rice J, Ottensmeier CH, Stevenson FK. DNA vaccines: precision tools for activating effective immunity against cancer. Nat Rev Cancer 2008; 8:108-20; PMID:18219306; http://dx.doi.org/10.1038/nrc2326.
5. Prud'homme GJ. DNA vaccination against tumors. J Gene Med 2005; 7:3-17; PMID:15538731; http://dx.doi.org/10.1002/jgm.669.
6. Iezzi M, Quaglino E, Amici A, Lollini PL, Forni G, Cavallo F. DNA vaccination against oncoantigens: A promise. Oncoimmunology 2012; 1:316-25; PMID:22737607; http://dx.doi.org/10.4161/onci.19127.
7. Cavallo F, Offringa R, van der Burg SH, Forni G, Melief CJ. Vaccination for treatment and prevention of cancer in animal models. Adv Immunol 2006; 90:175-213; PMID:16730264; http://dx.doi.org/10.1016/S0065-2776(06)90005-4.

8. Babiuk LA, Pontarollo R, Babiuk S, Loehr B, van Drunen Littel-van den Hurk S. Induction of immune responses by DNA vaccines in large animals. Vaccine 2003; 21:649-58; PMID:12531334; http://dx.doi.org/10.1016/S0264-410X(02)00574-1.
9. Steinhagen F, Kinjo T, Bode C, Klinman DM. TLR-based immune adjuvants. Vaccine 2011; 29:3341-55; PMID:20713100; http://dx.doi.org/10.1016/j.vaccine.2010.08.002.
10. Scheerlinck JY. Genetic adjuvants for DNA vaccines. Vaccine 2001; 19:2647-56; PMID:11257404; http://dx.doi.org/10.1016/S0264-410X(00)00495-3.
11. Greenland JR, Letvin NL. Chemical adjuvants for plasmid DNA vaccines. Vaccine 2007; 25:3731-41; PMID:17350735; http://dx.doi.org/10.1016/j.vaccine.2007.01.120.
12. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer into mouse muscle in vivo. Science 1990; 247:1465-8; PMID:1690918; http://dx.doi.org/10.1126/science.1690918.
13. Frelin L, Brass A, Ahlén G, Brenndörfer ED, Chen M, Sällberg M. Electroporation: a promising method for the nonviral delivery of DNA vaccines in humans? Drug News Perspect 2010; 23:647-53; PMID:21180650; http://dx.doi.org/10.1358/dnp.2010.23.10.1513492.
14. Hutnick NA, Myles DJF, Bian CB, Muthumani K, Weiner DB. Selected approaches for increasing HIV DNA vaccine immunogenicity in vivo. Curr Opin Virol 2011; 1:233-40; PMID:22440782; http://dx.doi.org/10.1016/j.coviro.2011.08.003.
15. Lin F, Shen X, McCoy JR, Mendoza JM, Yan J, Kemmerrer SV, et al. A novel prototype device for electroporation-enhanced DNA vaccine delivery simultaneously to both skin and muscle. Vaccine 2011; 29:6771-80; PMID:21199706; http://dx.doi.org/10.1016/j.vaccine.2010.12.057.
16. Vahlsing HL, Yankauckas MA, Sawdey M, Gromkowski SH, Manthorpe M. Immunization with plasmid DNA using a pneumatic gun. J Immunol Methods 1994; 175:11-22; PMID:7930633; http://dx.doi.org/10.1016/0022-1759(94)90327-1.
17. Haensler J, Verdelet C, Sanchez V, Girerd-Chambaz Y, Bonnin A, Trannoy E, et al. Intradermal DNA immunization by using jet-injectors in mice and monkeys. Vaccine 1999; 17:628-38; PMID:10067667; http://dx.doi.org/10.1016/S0264-410X(98)00242-4.
18. Sawamura D, Ina S, Itai K, Meng X, Kon A, Tamai K, et al. In vivo gene introduction into keratinocytes using jet injection. Gene Ther 1999; 6:1785-7; PMID:10516730; http://dx.doi.org/10.1038/sj.gt.3301002.
19. Bins AD, Jorritsma A, Wolkers MC, Hung CF, Wu TC, Schumacher TNM, et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. Nat Med 2005; 11:899-904; PMID:15965482; http://dx.doi.org/10.1038/nm1264.
20. Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD Jr. DNA-based immunization by in vivo transfection of dendritic cells. Nat Med 1996; 2:1122-8; PMID:8837611; http://dx.doi.org/10.1038/nm1096-1122.
21. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. J Exp Med 1998; 188:1075-82; PMID:9743526; http://dx.doi.org/10.1084/jem.188.6.1075.
22. Romani N, Holzmann S, Tripp CH, Koch F, Stoitzner P. Langerhans cells - dendritic cells of the epidermis. APMIS 2003; 111:725-40; PMID:12974775; http://dx.doi.org/10.1034/j.1600-0463.2003.11107805.x.
23. Bauer J, Bahmer FA, Wörl J, Neuhuber W, Schuler G, Fartasch M. A strikingly constant ratio exists between Langerhans cells and other epidermal cells in human skin. A stereologic study using the optical disector method and the confocal laser scanning microscope. J Invest Dermatol 2001; 116:313-8; PMID:11180009; http://dx.doi.org/10.1046/j.1523-1747.2001.01247.x.
24. Bergstresser PR, Fletcher CR, Streilein JW. Surface densities of Langerhans cells in relation to rodent epidermal sites with special immunologic properties. J Invest Dermatol 1980; 74:77-80; PMID:6985948; http://dx.doi.org/10.1111/1523-1747.ep12519909.
25. Lenz A, Heine M, Schuler G, Romani N. Human and murine dermis contain dendritic cells. Isolation by means of a novel method and phenotypical and functional characterization. J Clin Invest 1993; 92:2587-96; PMID:8254016.
26. Walther W, Stein U, Fichtner I, Malcherek L, Lemm M, Schlag PM. Nonviral in vivo gene delivery into tumors using a novel low volume jet-injection technology. Gene Ther 2001; 8:173-80; PMID:11313788; http://dx.doi.org/10.1038/sj.gt.3301350.
27. Walther W, Stein U, Lage H. Jet-injection of short hairpin RNA-encoding vectors into tumor cells. Methods Mol Biol 2010; 629:123-39; PMID:20387147; http://dx.doi.org/10.1007/978-1-60761-657-3_9.
28. Walther W, Stein U, Fichtner I, Kobelt D, Aumann J, Arlt F, et al. Nonviral jet-injection gene transfer for efficient in vivo cytosine deaminase suicide gene therapy of colon carcinoma. Mol Ther 2005; 12:1176-84; PMID:16202659; http://dx.doi.org/10.1016/j.ymthe.2005.07.700.
29. Walther W, Stein U, Fichtner I, Schlag PM. Low-volume jet injection for efficient nonviral in vivo gene transfer. Mol Biotechnol 2004; 28:121-8; PMID:15477651; http://dx.doi.org/10.1385/MB:28:2:121.
30. Walther W, Siegel R, Kobelt D, Knösel T, Dietel M, Bembenek A, et al. Novel jet-injection technology for nonviral intratumoral gene transfer in patients with melanoma and breast cancer. Clin Cancer Res 2008; 14:7545-53; PMID:19010873; http://dx.doi.org/10.1158/1078-0432.CCR-08-0412.
31. Ménard S, Pupa SM, Campiglio M, Tagliabue E. Biologic and therapeutic role of HER2 in cancer. Oncogene 2003; 22:6570-8; PMID:14528282; http://dx.doi.org/10.1038/sj.onc.1206779.
32. Curigliano G, Spitaleri G, Pietri E, Rescigno M, de Braud F, Cardillo A, et al. Breast cancer vaccines: a clinical reality or fairy tale? Ann Oncol 2006; 17:750-62; PMID:16293674; http://dx.doi.org/10.1093/annonc/mdj083.
33. Baxevanis CN, Voutsas IF, Gritzapis AD, Perez SA, Papamichail M. HER-2/neu as a target for cancer vaccines. Immunotherapy 2010; 2:213-26; PMID:20635929; http://dx.doi.org/10.2217/imt.09.89.
34. Peoples GE, Holmes JP, Hueman MT, Mittendorf EA, Amin A, Khoo S, et al. Combined clinical trial results of a HER2/neu (E75) vaccine for the prevention of recurrence in high-risk breast cancer patients: U.S. Military Cancer Institute Clinical Trials Group Study I-01 and I-02. Clin Cancer Res 2008; 14:797-803; PMID:18245541; http://dx.doi.org/10.1158/1078-0432.CCR-07-1448.
35. Nguyen-Hoai T, Baldenhofer G, Ahmed MS, Pham-Duc M, Gries M, Lipp M, et al. CCL19 (ELC) improves TH1-polarized immune responses and protective immunity in a murine Her2/neu DNA vaccination model. J Gene Med 2012; 14:128-37; PMID:22228591; http://dx.doi.org/10.1002/jgm.1651.
36. Nguyen-Hoai T, Baldenhofer G, Sayed Ahmed MS, Pham-Duc M, Vu MD, Lipp M, et al. CCL21 (SLC) improves tumor protection by a DNA vaccine in a Her2/neu mouse tumor model. Cancer Gene Ther 2012; 19:69-76; PMID:21997231; http://dx.doi.org/10.1038/cgt.2011.69.
37. Smorlesi A, Papalini F, Amici A, Orlando F, Pierpaoli S, Mancini C, et al. Evaluation of different plasmid DNA delivery systems for immunization against HER2/neu in a transgenic murine model of mammary carcinoma. Vaccine 2006; 24:1766-75; PMID:16288939; http://dx.doi.org/10.1016/j.vaccine.2005.10.022.
38. Lindencrona JA, Preiss S, Kammertoens T, Schüler T, Piechocki M, Wei WZ, et al. CD4+ T cell-mediated HER-2/neu-specific tumor rejection in the absence of B cells. Int J Cancer 2004; 109:259-64; PMID:14750178; http://dx.doi.org/10.1002/ijc.11654.
39. Greenberg PD, Cheever MA, Fefer A. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1+2- lymphocytes. J Exp Med 1981; 154:952-63; PMID:6974221; http://dx.doi.org/10.1084/jem.154.3.952.
40. Hanson HL, Donermeyer DL, Ikeda H, White JM, Shankaran V, Old LJ, et al. Eradication of established tumors by CD8+ T cell adoptive immunotherapy. Immunity 2000; 13:265-76; PMID:10981969; http://dx.doi.org/10.1016/S1074-7613(00)00026-1.
41. Trimble C, Lin CT, Hung CF, Pai S, Juang J, He L, et al. Comparison of the CD8+ T cell responses and antitumor effects generated by DNA vaccine administered through gene gun, biojector, and syringe. Vaccine 2003; 21:4036-42; PMID:12922140; http://dx.doi.org/10.1016/S0264-410X(03)00275-5.
42. Raviprakash K, Porter KR. Needle-free injection of DNA vaccines: a brief overview and methodology. Methods Mol Med 2006; 127:83-9; PMID:16988448.
43. Janouskova O, Nellessen T, Stokrova J, Jinoch P, Smahel M. Delivery of recombinant adeno-associated virus by jet injection. Int J Mol Med 2003; 12:687-91; PMID:14532994.
44. Kanazawa T, Takashima Y, Tamura T, Tsuchiya M, Shibata Y, Udagawa H, et al. Local gene expression and immune responses of vaginal DNA vaccination using a needle-free injector. Int J Pharm 2010; 396:11-6; PMID:20621704; http://dx.doi.org/10.1016/j.ijpharm.2010.05.040.
45. Imoto J, Konishi E. Needle-free jet injection of a mixture of Japanese encephalitis DNA and protein vaccines: a strategy to effectively enhance immunogenicity of the DNA vaccine in a murine model. Viral Immunol 2005; 18:205-12; PMID:15802965; http://dx.doi.org/10.1089/vim.2005.18.205.
46. Simon JK, Carter M, Pasetti MF, Sztein MB, Kotloff KL, Weniger BG, et al. Safety, tolerability, and immunogenicity of inactivated trivalent seasonal influenza vaccine administered with a needle-free disposable-syringe jet injector. Vaccine 2011; 29:9544-50; PMID:21986218; http://dx.doi.org/10.1016/j.vaccine.2011.09.097.
47. Mitragotri S. Current status and future prospects of needle-free liquid jet injectors. Nat Rev Drug Discov 2006; 5:543-8; PMID:16816837.
48. Ren S, Li M, Smith JM, De Tolla LJ, Furth PA. Low-volume jet injection for intradermal immunization in rabbits. BMC Biotechnol 2002; PMID:12028591; 2:10.http://dx.doi.org/10.1186/1472-6750-2-10.
49. Schramm-Baxter J, Mitragotri S. Needle-free jet injections: dependence of jet penetration and dispersion in the skin on jet power. J Control Release 2004; 97:527-35; PMID:15212884; http://dx.doi.org/10.1016/j.jconrel.2004.04.006.
50. Stoecklinger A, Grieshuber I, Scheiblhofer S, Weiss R, Ritter U, Kissenpfennig A, et al. Epidermal langerhans cells are dispensable for humoral and cell-mediated immunity elicited by gene gun immunization. J Immunol 2007; 179:886-93; PMID:17617579.
51. Stoecklinger A, Eticha TD, Mesdaghi M, Kissenpfennig A, Malissen B, Thalhamer J, et al. Langerin+ dermal dendritic cells are critical for CD8+ T cell activation and IgH γ-1 class switching in response to gene gun vaccines. J Immunol 2011; 186:1377-83; PMID:21187444; http://dx.doi.org/10.4049/jimmunol.1002557.

52. Moser M. Dendritic cells in immunity and tolerance-do they display opposite functions? Immunity 2003; 19:5-8; PMID:12871634; http://dx.doi.org/10.1016/S1074-7613(03)00182-1.
53. Kalinski P, Moser M. Consensual immunity: success-driven development of T-helper-1 and T-helper-2 responses. Nat Rev Immunol 2005; 5:251-60; PMID:15738955; http://dx.doi.org/10.1038/nri1569.
54. Speiser DE, Romero P. Molecularly defined vaccines for cancer immunotherapy, and protective T cell immunity. Semin Immunol 2010; 22:144-54; PMID:20413326; http://dx.doi.org/10.1016/j.smim.2010.03.004.
55. Ikeda H, Chamoto K, Tsuji T, Suzuki Y, Wakita D, Takeshima T, et al. The critical role of type-1 innate and acquired immunity in tumor immunotherapy. Cancer Sci 2004; 95:697-703; PMID:15471553; http://dx.doi.org/10.1111/j.1349-7006.2004.tb03248.x.
56. Ma Y, Aymeric L, Locher C, Kroemer G, Zitvogel L. The dendritic cell-tumor cross-talk in cancer. Curr Opin Immunol 2011; 23:146-52; PMID:20970973; http://dx.doi.org/10.1016/j.coi.2010.09.008.
57. Chen Y, Webster RG, Woodland DL. Induction of CD8+ T cell responses to dominant and subdominant epitopes and protective immunity to Sendai virus infection by DNA vaccination. J Immunol 1998; 160:2425-32; PMID:9498786.
58. Curcio C, Di Carlo E, Clynes R, Smyth MJ, Boggio K, Quaglino E, et al. Nonredundant roles of antibody, cytokines, and perforin in the eradication of established Her-2/neu carcinomas. J Clin Invest 2003; 111:1161-70; PMID:12697735.
59. Nagata Y, Furugen R, Hiasa A, Ikeda H, Ohta N, Furukawa K, et al. Peptides derived from a wild-type murine proto-oncogene c-erbB-2/HER2/neu can induce CTL and tumor suppression in syngeneic hosts. J Immunol 1997; 159:1336-43; PMID:9233630.
60. Piechocki MP, Pilon SA, Wei WZ. Quantitative measurement of anti-ErbB-2 antibody by flow cytometry and ELISA. J Immunol Methods 2002; 259:33-42; PMID:11730839; http://dx.doi.org/10.1016/S0022-1759(01)00487-2.