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# The impact of frost-damage on the quality and quantity of the secreted antigen-specific IgG repertoire

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ABSTRACT

Freezing of alum-based vaccines drastically alters their colloidal composition and leads to irreversible cluster formation. The loss of stability is well described, but the impact of frost damage on the functionality of the induced and secreted antibody repertoire has not been studied in detail. We therefore applied our single-cell measurement platform to extract the frequencies of Immunoglobulin G-secreting cells in combination with individual secretion rates and affinities. We showed that, frost-damaged or not, the tested vaccine was able to generate similar frequencies of total and antigen-affine IgG-secreting cells. Additionally, the frost-damaged vaccine stimulated a similar T-cell cytokine secretion pattern when compared to the regularly stored vaccine. However, frost-damaged vaccines induced no efficient affinity maturation and a complete collapse of the affinity distribution was observed. This study unveiled the impact of frost-damage to alum-based vaccines on the induced secreted antibody repertoire, and illustrated the power of functional single-antibody analysis.



## 1. Introduction

Vaccines have had and continue to have a tremendous positive effect on global health [1–3]. Preventive in their action, they are able to protect recipients from specific disease due to the induced preparative immunological response. In many cases, the present antigens lead to the generation of a long-lasting antigen-specific antibody repertoire cells [4–6], and long-term protection is achieved due to the continued presence of antibodies within the organism and the development of immunological memory [7]. To induce a lasting and significant response, a successful vaccine integrates the antigenic parts into a suitable formulation that enables the generation of immunological memory and sustained antibody production [8–11]. Today, the most commonly used adjuvant for applications in humans is aluminum hydroxide (alum) [12]. While safe and efficient, this colloidal formulation was reported to be unstable when exposed to freezing temperatures, resulting in the irreversible formation of clusters [13,14]. Although this risk is well-described and known, reviews regularly found and still find that exposure to freezing temperatures is not an exception [15,16], potentially resulting in frost damaged vaccines [17–19]. While the impact of vaccine freezing on the colloidal suspension and the vaccine composition has been well-studied, much less focus has been laid on the influence of these altered vaccines on the quantity, quality and distribution of the generated functional Immunoglobulin-G repertoire. These class-switched highly affine antibodies are of great importance when assaying vaccine protection. We therefore applied our recently described technology ('DropMap') [20,21], a technology that enables us to measure the IgG-repertoire with single-antibody resolution, to characterize and quantitatively describe the immune reaction induced by immunization using freeze-damaged and properly stored model vaccine in mice (Fig. 1A). In this technology, individual cells were compartmentalized in 50 pL droplets and their secretion is analyzed over time using fluorescence microscopy. Due to the isolation and small volume within the droplet (50 pL), any secreted product from the cells was contained, its dilution minimized, controlled and a measurable concentration of the secreted molecule was reached in a short time frame (minutes to 1 h). For measurements, these droplets were arrayed in 2-dimensions, and the fluorescence relocation was read over time on an epi-fluorescent microscope, enabling to kinetically study the behavior of around 1′000-200′000 cells per run. Each droplet further contained an immunoassay based on fluorescence relocation able to quantita-

*Abbreviations:* TT, tetanus toxoid; DT, diphtheria toxoid; DTP, diphtheria tetanus poliomyelitis; IgG-SCs, Immunoglobulin-G secreting cells; $K^D$, dissociation constant.

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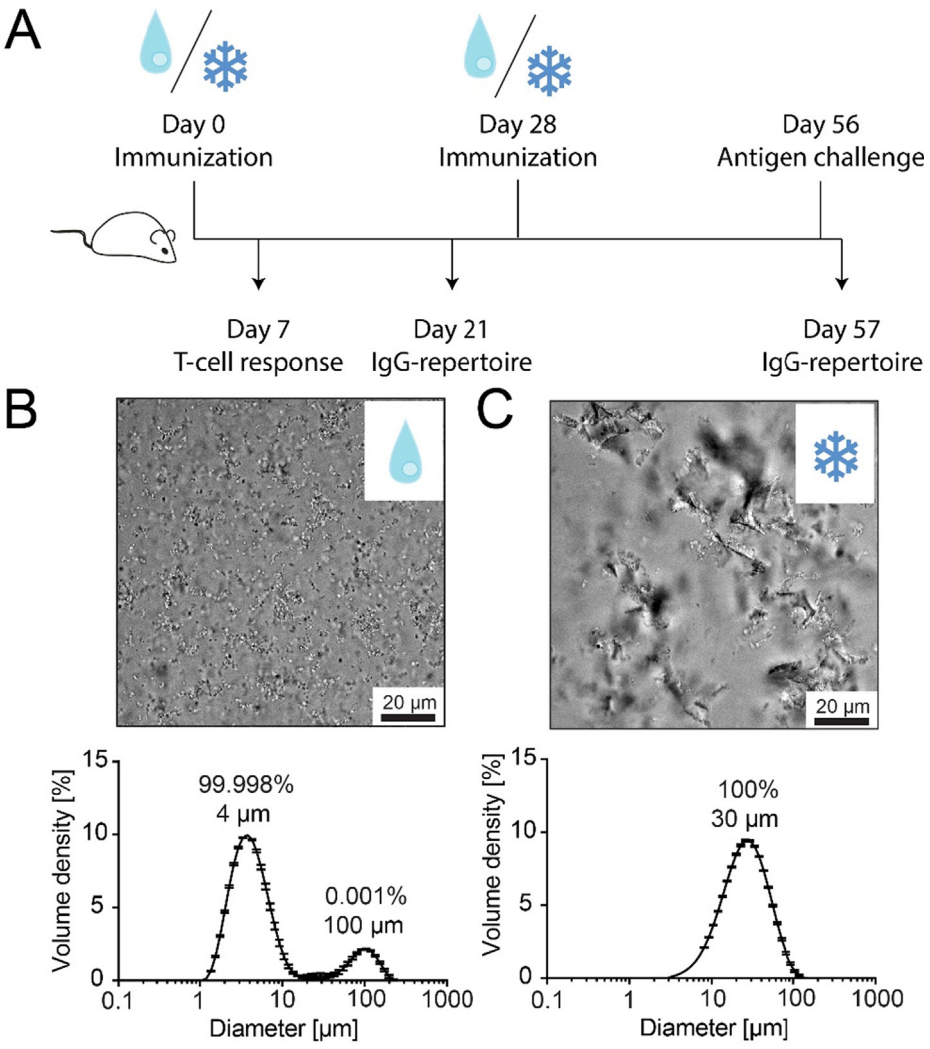


**Fig. 1.** (A) Overview of the immunization schedule with specified days of analysis. (B&C) Microscopic images and particle size distribution of properly stored (B) and shortly frozen (<1 h, (C) commercial DTP vaccine. Percentage in the figure represent the number of events. Static light scattering experiments revealed the presence of one uniform population of alum particles when the vaccine was stored according to the manufacturer's instructions (B). However, when shortly frozen, particles were found to have collapsed to bigger structures with sizes around 30 µm (C).

tively study IgG secretion [20,21]. The kinetic measurement in combination with the herein used bioassay allowed to precisely measure the frequency of secreting cells and their individual secretion rates, but also the frequency of antigen-affine immunoglobulin-G secreting cells (IgG-SCs) and the affinity of the secreted antibodies towards given antigens (for dissociation constants $K_D$ < 500 nM). Such a system is especially powerful when analyzing IgG-SCs since each cell only secretes one type of antibody at a given time [22,23], enabling the analysis of the secreted antibody repertoire with single-antibody resolution. Therefore, this approach allowed quantitative analysis of the secreted antibody repertoire in a short amount of time with high throughput and resolution. Due to the quantitative aspect of our technology, the system enabled us therefore to measure cellular frequencies, secretion rates as well as affinities with single-cell and antibody resolution, enabling a much better characterization of the immune reaction than a titer measurement. To study the impact of frost-damage, we decided to analyze the induced IgG-repertoire at various times after immunization in order to evaluate the impact on generation, maturation, selection and transfer [21]. As a model vaccine, we used Revaxis (Sanofi Pasteur), a diphtheria-tetanus-polio myelitis vaccine (DTP) that is known to be frost-sensitive [17].

## 2. Methods

**Immunization of mice.** After two weeks of acclimatisation, BALB-C mice (Janvier Labs, female, age 6–8 weeks) were immunized intraperitoneal with 100 µl Revaxis (Sanofi Pasteur), 100 µl of frost-damaged Revaxis or the indicated amount of tetanus toxoid (TT, Statens serum Institute, Copenhagen) in Alhydrogel® adjuvant 2% (alum, InvivoGen) or complete Freud's adjuvants (CFA, Sigma Aldrich) according to the schedule (Fig. 1A). Due to ethical concerns, practical limitations and data comparison with previous experiments, *i.p.* immunization was favoured. For immunizations with frozen vaccine, the vaccine was put for 1 h at −20 °C, subsequently thawed and applied at 100 µl per mouse and injection. No alterations in behaviour or additional pain were observed in mice immunized with frost-damaged vaccine. All immunizations and experiments were performed in triplicates. At indicated days, the mice were killed, splenocytes were extracted and prepared for encapsulation as described in reference [21]. Experiments using mice were validated by the CETEA ethics committee number 89 (Institute Pasteur, Paris, France) under #2013-0103, and by the French Ministry of Research under agreement #00513.02; and are part of a larger study.

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**DropMap measurements**. For the methods to prepare the observation chamber, the droplet generator, magnetic nanoparticles for signal detection as well as approach for the data acquisition and analysis please refer to reference [20,24]. As for detection, in-house Alexa488-labelled purified tetanus toxoid (Statens serum, Copenhagen) and Alexa555-labelled diphtheria toxin (Glu52-variant, S2189, Sigma-Aldrich), each at a final concentration of 40 nM as well as the Alexa647-labelled anti-IgG (Jackson ImmunoResearch) at a final in-droplet concentration of 75 nM were used. Data analysis was performed as described else-where [20]. If not mentioned otherwise, median and standard error of the mean are indicated. For statistical analysis, GraphPad prism 8 was used, and two-tailed t-tests were used to test for significance. Distributions are shown as kernel probability plots fitted by Matlab (fitted histograms with infinitely small bin sizes).

**Cytokine measurements.** For cytokine secretion experiments, spleens of immunized mice were harvested 7 days after primary injection. The cells were further processed according to the manufacturer's protocol using the Pan T Cell Isolation Kit II on a MultiMACS Cell24 Separator Plus (both Miltenyi). Afterwards, cells were collected, washed once by centrifugation (300g, 5 min), and re-suspended at a concentration of 2 Mio/ml. 100 µl of T-cell suspension was incubated for 4 h at 37 °C in TexMACS media (Miltenyi) supplemented with 1% Penicillin/Streptomycin (ThermoFisher). Cells and debris were pelleted at 2000 g; and the supernatant was stored at −80 °C until measurement. To assess cytokine secretion, a commercial Th1/Th2/Th17 multiplex immunoassay kit (Fireplex, ab213395, Abcam) was used according to the manufacturer's manual. For measurements, the particles were incubated with the collected cell supernatants as described in the manufacturer's protocol; and subsequently passed on the Sony SH800 FACS Cell sorter (Sony). Analysis of data was performed with the Fireplex Analysis Workbench (Abcam).

**Static light scattering and light microscopy.** Static light scattering experiments were performed using a Malvern Mastersizer 3000 on frost-damaged as well as properly stored vaccine. As for light microscopy, we used a Nikon Ti2 inverted microscope.

## 3. Results and discussion

**Particle distribution of fresh and frozen commercial DTP vaccine confirmed the impact of freezing.** Microscopic and static light scattering (SLS) analysis of properly stored commercial DTP vaccine and frost damaged DTP vaccine confirmed the impact of the short exposure to freezing temperatures. Properly stored DTP vaccine showed a size distribution centered around 3–5 µm (Fig. 1B, left top), after freezing for 1 h and subsequent thawing the particle distribution peaked around 30 µm (Fig. 1C, right top), a size similar to the one described in literature [13,14]. Light microscopy confirmed the presence of large irregular aggregates (Fig. 1B and C).

**Frost-damaged DTP vaccines induced qualitative defects in the functional IgG-repertoire.** In order to assay the repertoire with individual antibody resolution, we immunized Balb/C mice with properly stored and frost-damaged vaccine and assessed the frequencies of total as well as TT and DT-affine IgG-SCs, their secretion rates and affinities 21 days after primary immunization (Fig. 1A). At this time, affinity maturation and clonal expansion have already occurred within the spleen, and the organ still contained considerable frequencies of IgG-SCs from the previous immunization [20,21]. Interestingly, both immunizations were able to induce non-significantly different frequencies of total and antigen-specific IgG-SCs (Fig. 2A, total IgG-SCs 0.39 ± 0.14 and 0.39 ± 0.04%, TT-affine 0.14 ± 0.07 and 0.11 ± 0.02%, DT-affine 0.18 ± 0.05 and 0.15 ± 0.04%, $p > 0.4$ for all, $n = 3$ for all, mean ± STDEV). Additionally, the median secretion rates were non-significantly different as well (Fig. 2B, 24 ± 7 and 28 ± 7 IgG/s, $p = 0.5$, $n = 3$, mean ± STDEV), and the single-cell distribution of the assayed secretion rate showed an almost complete overlap between the two conditions (Fig. 2D). In contrast to these similarities, the impact of frost damage was best illustrated by comparing the median affinities of anti-TT cells. Observed median affinities significantly collapsed upon the use of frost-damaged vaccine (Fig. 2C, 120 ± 33 nM vs. 48 ± 23 nM, $p = 0.03$), and the distributions showed the full impact of using a freeze-damaged vaccine (Fig. 2E). Whereas most of the TT-affine IgG-SCs induced by properly stored DTP-vaccine displayed dissociation constants ($K_D$) lower than 100 nM (around 80%), this frequency was remarkably lower in frost-damaged immunizations (20%). The distribution for frost-damaged immunized mice was characterized by much broader range of measured affinities, and an increased frequency of IgG-SCs displaying low affinities between 100 and 350 nM $K_D$s. The most dramatic observation was the almost complete absence of high-affinity cells (in the category $K_D < 10$ nM, 23.5% and 1.8%, respectively) in the immunizations performed with the frost-damaged DTP-vaccine.

Furthermore, we measured the IgG-repertoire after a complete immunization schedule with two immunizations each four weeks apart; and a recall booster with pure antigen as challenge (Fig. 1A) [21]. At this point, we were assaying the recall responsive IgG repertoire that would be available upon future possible antigen encounters [21]. The two injections were either performed with properly stored or frost damaged vaccines and preceded a recall containing pure antigen (TT). Different sequences of properly stored or frost damaged vaccines were assayed to measure the impact of a frost-damaged vaccine within an immunization schedule. First experiments were performed with two immunizations using properly stored or frost-damaged vaccine. Here, the frequency of IgG-SCs was found to be significantly lower when frost-damaged vaccine was used (0.63 ± 0.02% vs 1.08 ± 0.17%, $p < 0.001$, Fig. 3A). This finding indicated that although both immunizations generated similar frequencies, their ability to induce immunological memory and long-lived plasma cells was different. However, no significant difference was detected in the frequency of antigen-specific cells (defined by $K_D < 500$ nM), both TT- and DT-affine IgG-SCs ($p > 0.1$). Similar to earlier time points though (Fig. 2), median secretion rate (Fig. 3B) as well as the distribution thereof did not show any important difference (Fig. 3D, except for the frequency, $p = 0.41$). Furthermore, likewise our previous results have demonstrated (Fig. 2), immunizations schedules with only frost-damaged vaccines heavily influenced measured affinities, both median and the distributions thereof (Fig. 3C and E). Significantly lower affinities were assessed for anti-TT and anti-DT antibodies when frost-damaged vaccines were used (for anti-TT 59 ± 3 nM vs. 135 ± 3 nM, p-value < 0.001; $n = 3$, for anti-DT 37 ± 9 nM vs. 112 ± 8 nM, $n = 3$, p value < 0.001). Indeed, when comparing the affinity distributions instead of median values (Fig. 3E), immunizations with defrosted vaccine resulted in a broader distribution over the whole affinity range, as well as a decreased frequency of high-affinity cells for both assayed antigens (9.7% and 0.7% for anti-TT IgGs, 23.5% and 4.69% for anti-DT IgGs, 5–15-fold reduction).

**Frost-damaged DTP vaccines induced a similar T-cell response.** After having confirmed the effect of freezing on the vaccine formulation, we were interested whether the application of frozen vaccines was able to mount a proper T helper response. By measuring the respective cytokine secretion patterns (see Methods for detail), we found significant differences in cytokine production between the DTP vaccine and various other injections with TT alone or combined with different adjuvants, what indicated that the applied method was able to assess different cytokine

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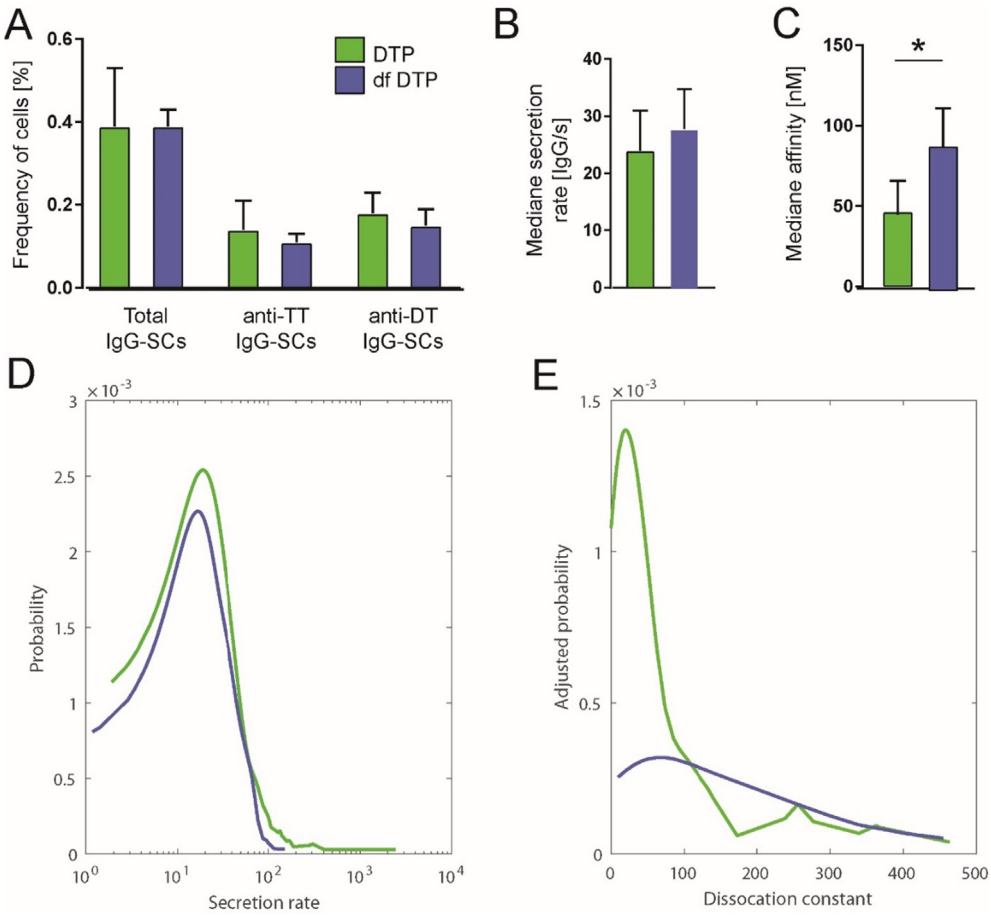


**Fig. 2.** Secreted IgG repertoire within the spleen 21 days after immunization. (A) Frequencies of total IgG-SCs, TT- and DT affine IgG-SCs (as defined as $K_D$ < 500 nM). DTP represents properly stored vaccine, dfDTP frost-damaged vaccine. From A-C, data is shown as average and standard deviation over three mice. (B) Median secretion rates of all present cells. (C) Median $K_D$ extracted from the individual mice. (D) Kernel probability distributions of the individual secretion rates. Data from the three experiments were binned and fitted with a Kernel probability distribution (see Methods). (E) Kernel probability distributions of the measured dissociation constants for TT-affine cells. DT affine data was found to be similar in their distribution (data not shown). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

expression levels (Fig. 4A). Nevertheless, the alum-based DTP vaccine, frost-damaged or not, induced a very similar cytokine secretion pattern. Significant differences were found only for IL-10 (about 4-fold decrease for the frost-damaged vaccine, $p < 0.05$). Therefore, the general cytokine response remained strikingly similar.

**Frost-damage reduced the available antigen dose.** Next, we were interested in observing the possible impact of antigen bioavailability on the reaction. We therefore prepared in house immunizations with alum as adjuvant and varying TT doses, and measured median affinities in mice. As demonstrated in Fig. 4B, median affinity nicely followed a dose-response trend ($R^2 = 0.98$). Even the immunization with Revaxis with an injected dose of around 4 μg per mouse and per injection (i.e. 2 Lf) nicely agreed with this model. Therefore, a measured median affinity of 135 ± 3 nM in frost-damaged vaccines corresponds to an available dose of 0.3 ± 0.02 μg TT, representing a 13-fold reduction in available dose.

## 4. Conclusion

In this study, we measured the influence of frost damage on the DTP vaccine-induced antibody repertoire. Interestingly, the frequencies of total IgG-SCs and the distribution of antibody secretion rates were not significantly affected by frost-damage, even for antigen-specific IgG-SCs. However, the impact of frost-damage was best illustrated by the collapse of the measured affinity distribution and its median affinity. The data revealed an almost complete absence of high-affinity IgG-SCs. Although the median affinity for frost-damaged vaccine was significantly different from the one induced upon immunizations with alum only (without antigen, $p < 0.01$), this frost-damaged formulation was no longer able to induce affinity maturation and correlated to/corresponded to an about 13-fold reduction of available dose. Therefore, the absence of detectable titers in mice immunized with frost-damaged vaccines was mostly due to the absence of high-affinity IgGs, whereas low frequencies of low affinity IgGs were still generated by the immunization.

In contrast, the application of frost-damaged vaccines resulted in similar T cell cytokine secretion pattern. The only significant difference in cytokine secretion was found for IL-10, a cytokine that plays and important immune-regulatory function and is shaping adaptive immune responses due to its influence on Th1/Th2 balance [25,26]. A high concentration of IL-10 was correlated with the presence of a Th2 response and should result in decreased secretion of pro-inflammatory cytokine. Indeed, consistently lower levels of these pro-inflammatory cytokines were found in mice immunized with properly stored vaccine, although not at a significant level. Here, additional experiments are needed to study the cytokine secretion and decipher the impact of frost-damage on the Th1/Th2 balance, and its impact on the secreted antibody repertoire.

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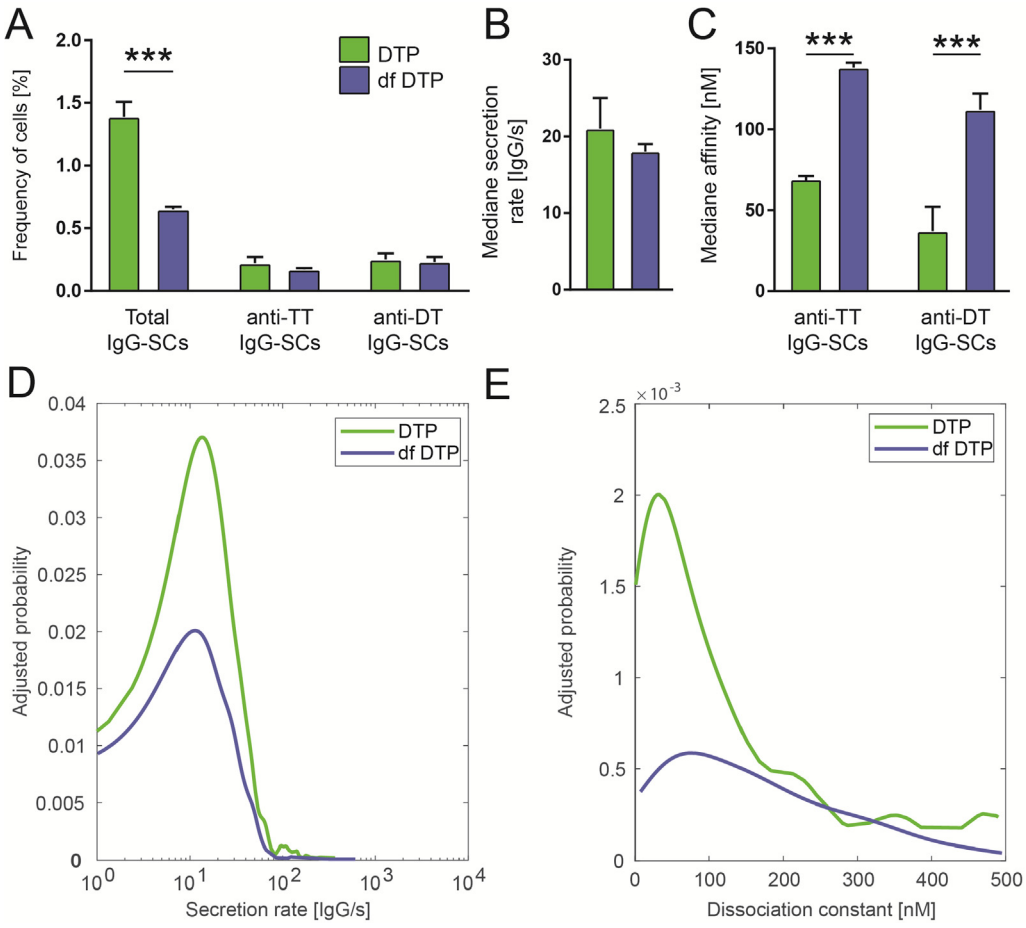


**Fig. 3.** Secreted IgG repertoire within the spleen day 56, I.e. 24 h after TT-challenge. (A) Frequencies of total IgG-SCs, TT- and DT affine IgG-SCs ($K_D$ < 500 nM). (B) Median secretion rates. (C) Median $K_D$. (D) Kernel probability distributions of the individual secretion rates. (E) Kernel probability distributions of the measured dissociation constants for TT affine IgGs in properly stored (green) and frost-damaged vaccines (dark blue, df DTP). * p < 0.05, ** p < 0.01, *** p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

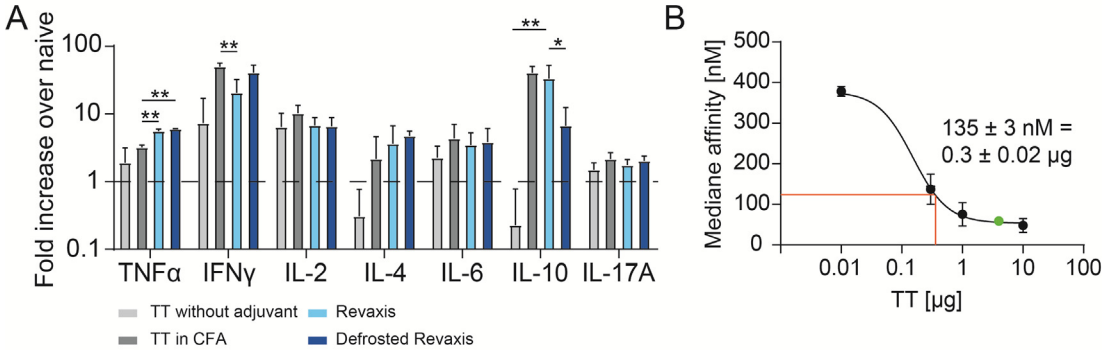


**Fig. 4.** (A) Cytokine secretion by splenic T-cells 7 days after immunization with properly stored (light blue) and frost-damaged (dark blue) DTP vaccine. Cytokine secretion profiles are comparable; the only significant difference was found in IL-10 production. For comparison, cytokine data for immunization with TT alone (light grey) and TT with CFA (dark grey) are shown. All cytokine levels were significantly different from the immunizations with TT alone and naïve mice (not shown for visibility). * p < 0.05, ** p < 0.01, *** p < 0.001. (B) Calibration curve for the extracted median affinity and TT alum immunizations. Data points represent the average median affinity and standard deviation over three immunized and analyzed mice. Data points in black refer to immunizations prepared in house, green to properly stored Revaxis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Different mechanisms could be responsible for the absence of the high-affinity IgG-SCs. The increased particle size was described to limit the interaction and transport of these particles by antigen-presenting cells [27]. This would result in a lower bioavailability of the injected dose, as well as lower antigen amounts present during affinity maturation [21]. Suboptimal amounts of antigen were described to lead to low affinity IgG-SCs [21]. Alternatively, the epitopes on the antigens themselves could also be affected by freezing [28], or the above described change in Th1/Th2 balance could influence the antibody repertoire. Other mechanisms also remain possible, and additional studies will need to further characterize and decipher the influence of each of these parameters.

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Our study shows the impact of immunization with frost-damaged vaccines on the antibody repertoire in a murine model system. Whilst the results obtained in this model system are clear and significant, we cannot conclude on the transferability of these results to a human frame. However, our findings indicate that it is evidently important to respect the cold-chain and not expose vaccines to sub-optimal conditions [13–16]. DropMap, the technology employed in this study, has the resolution necessary to investigate the impact of voluntary or accidental changes to vaccines; and to decipher the impact of adjuvant, additives and antigens on the generated antibody repertoire with high biochemical resolution (affinity, specificity, secretion rates) at the single-antibody level.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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