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Formation of Immune Complexes with a Tetanus-Derived B Cell Epitope Boosts Human T Cell Responses to Covalently Linked Peptides in an Ex Vivo Blood Loop System

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Enhancing T cell responses against both viral and tumor Ags requires efficient costimulation and directed delivery of peptide Ags into APCs. Long peptide vaccines are considered favorable vaccine moieties from a clinical perspective, as they can harbor more than one immunogenic epitope enabling treatment of a broader target population. In addition, longer peptides are not extracellularly loaded on MHC class I; rather, they require intracellular processing and will thereby be presented to T cells mainly by professional APCs, thereby avoiding the risk of tolerance induction. The drawback of peptide vaccines regardless of peptide length is that naked peptides are not actively targeted to and taken up by APCs, and the standard nonconjugated adjuvant-peptide mixtures do not ensure cotargeting of the two to the same APC. We have identified a tetanus toxin–derived B cell epitope that can mediate the formation of immune complexes in the presence of circulating Abs. In this study, we show that these immune complexes improve both Ag uptake by APCs (blood monocytes and CD1c$^+$ dendritic cells) and consequently improve CD8$^+$ T cell recall responses in a human ex vivo blood loop system. The uptake of the peptide conjugate by blood monocytes is dependent on Abs and the complement component C1q. We envision that this strategy can be used to facilitate active uptake of Ags into APCs to improve T cell responses against pathogens or cancer. ***The Journal of Immunology*****, 2018, 201: 87–97.**

Dendritic cells (DCs) are one of the most important cell types of the immune system. They capture, process, and present Ags to both CD4$^+$ and CD8$^+$ T cells, which subsequently elicit an Ag-specific immune response. The capacity of DCs to drive an immune response toward Ag-specific T cell responses has spurred numerous clinical trials involving DCs in cancer immunotherapy. To boost antitumor responses, DCs are required to capture tumor Ags and receive sufficient activation stimuli. One strategy has been to load autologous DCs with tumor Ags ex vivo and activate them with cytokines before reinfusion (1). This strategy consists of laborious and expensive ex vivo culturing of DCs. Another strategy has been to vaccinate with one or several short peptides derived from tumor-associated Ags. The single-peptide vaccine is, however, strictly HLA-oriented and may induce tolerance as CTL priming occurs without help and in nonsecondary lymphoid organs (2–4). Vaccination using synthetic long peptides (SLPs) has emerged as a solution that would reduce the problem of tolerance induction as Ag presentation is steered to APCs, as these peptides require cellular uptake and intracellular processing (5). In addition, longer peptide stretches can harbor multiple epitopes along with a mix of CD8 and CD4 epitopes, enabling a more durable immune response (5). SLP vaccination has shown promising results in two clinical trials including patients with HPV-induced cancers (6, 7). However, further improvements can be made in regard to enhanced uptake and optimal stimulation of DCs. Work by ourselves and others (using Abs to enhance Ag uptake) has revealed a potency of immune complexes (ICs) to efficiently load DCs both in vitro and in vivo (8, 9). Additionally, these ICs can induce DC activation because FcγR cross-linking (10, 11) and priming of T cell responses is substantially enhanced, compared with responses when using naked Ag (9, 12, 13). Collectively, these characteristics of Ag-specific Abs are the reasons for the emerging idea of using them to target tumor Ags to DCs. We have previously demonstrated that circulating Ag-specific Abs can form Ag–Ab complexes to enhance priming and expansion of CD8$^+$ T cells in vivo after vaccination with a haptenated Ag (13). Recently, it has been shown that tumor Ag–specific mAbs induce T cell responses through the formation of ICs that have the capacity to improve cross-presentation and thereby long-lived antitumor responses (14).

To improve responses to tumor-associated Ags, one can envision the use of B cell epitopes such as haptens, sugars, or peptides as targets for circulating Abs to bind to. In this study, we make use of an 18-mer peptide from tetanus toxin to which IgG, rather than

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Abbreviations used in this article: AP, alternative pathway; CP, classical pathway; DC, dendritic cell; DTP, diphtheria, tetanus, and pertussis; IC, immune complex; raMTTE, rabbit anti-MTTE; raOVA, rabbit anti-OVA; SLP, synthetic long peptide; TTd, tetanus toxoid.

IgM, Abs bind. Our strategy aims to improve Ag uptake by active transportation and to promote cross-presentation. As SLPs are superior compared with protein in the intracellular routing and MHC loading (15), and as Ab–Ag complexes are stored in DCs to prolong Ag presentation (16), the combination of these two findings support our strategy. The identified B cell epitope (referred to as MTTE) is derived from tetanus toxin and was identified through a library peptide screen (17). In murine model systems, an $[MTTE]_3$-SLP conjugate improves DC activation as well as T cell priming in the presence of anti-MTTE–specific Abs (17).

In this work, we further evaluate the potential clinical use of our peptide conjugate in human blood using an ex vivo blood loop system (18). This system includes fresh human blood in circulation. By introducing our conjugates into the blood, we can study various biological parameters ex vivo. This system has retained intact complement activity, which allows interaction studies of Ag–Ab complexes with human whole blood and complement cascade system.

Materials and Methods

Reagents

OVA-Alexa Fluor 488 (OVA) was purchased from Invitrogen, and peptide conjugates were made with various SLPs (Table I). This was performed through maleimide coupling (of the MTTE/ETTM peptides) and copper-free click chemistry (free SLP or AFKSKACB-Alexa Fluor 488) as described in (17) using a model SLP. In short, three copies of the MTTE or ETTM peptide (ETTM is a scrambled MTTE control peptide, for all sequences see Table I), C-terminally extended with a spacer sequence (see Table I), are coupled to a core structure (for chemical structure see Ref. 17). A subsequent peptide (SLP or similar) that includes an azide group is coupled to the $[MTTE]_3$-core molecule to end up with the final peptide conjugates used in this study. Rabbit anti-MTTE (raMTTE) Abs were made by immunization of rabbits using the peptide MTTE conjugated to tetanus toxoid (TTd) administrated in CFA (prime) and IFA (boost). From isolated serum, Abs were purified using a protein G column. A protein G fraction of rabbit anti-OVA (raOVA) Abs were used together with OVA (Worthington Biochemical, Lakewood, NJ). FcγR-blocking Abs were as follows: anti-CD16 (Clone 3G8), anti-CD32 (Clone 7.3), and anti-CD64 (Clone 10.1) were obtained from Ancell. EGTA was purchased from Sigma-Aldrich. Compstatin (AcICV(1MeW)QDWGAHRCT) (19) was a kind gift from Prof. J.D. Lambris (University of Pennsylvania, School of Medicine). The C1q Ab (Clone JL-1) was purchased from Hycult Biotech. TetaQuin was purchased from Sanquin (Netherlands) and TTd from the Serum Institute (Denmark).

Hybridoma cell lines producing mouse anti-MTTE IgG1 and IgG2a were made by conjugating FIGITELKKLESKINKVFC-amide to keyhole limpet hemocyanin and through immunization of AIP-3 mice. When sufficient IgG1 and IgG2a titers were established, isolated spleen cells were fused with NS-1 myeloma cells. Primary clones and sub clones were analyzed for reactivity and two clones (one IgG1 and one IgG2a clone) were chosen for further Ab isolation. To isolate Abs, the hybridomas were cultured in CELLine 1000 bioreactors (Integra). The medium compartment contained DMEM/F-12 with 1% penicillin/streptomycin and 5% FBS (Ultra Low IgG FBS from Invitrogen), and the cell compartment contained DMEM/F-12 with 1% PEST, 2.5% FBS (Ultra Low IgG FBS from Invitrogen), and medium with high glucose (17.3 mM glucose and L-glutamine 2.5 mM). Harvested supernatants were spun down at 2500 rpm for 5 min and frozen at −80°C. Abs were purified on a protein G column (Capra Science).

Ag–Ab complex formation

Ag–Ab complexes were preformed in vitro by incubating Ag and Ab at 37°C for 30 min in serum-free medium. The final concentrations in blood were, unless otherwise stated, OVA (23 nM), raOVA (333 nM), $[MTTE]_3$-Alexa Fluor 488 (8 nM), and raMTTE (195 nM). The preformed complexes were stored at 4°C overnight before they were used in blood loop experiments.

Blood loop assay

Blood from healthy donors was taken in an open system and immediately mixed with the anticoagulant Lepirudin (Refludan) at the final concentration of 50 μg/ml. All materials in direct contact with the blood were surface heparinized with a heparin-conjugate from Corline AB (Sweden). Blood and conjugates were applied to heparinized polyvinyl chloride tubings from Corline, and the loops were sealed using specialized metal connectors. The blood loops were rotated on a wheel within a 37°C incubator. Blocking analyses were performed by incubating blood with blocking Abs (50 μg/ml), peptides (100 μg/ml), EGTA (5 mM), EDTA (2.5 mM), or Compstatin (10 μM) at 37°C for 10 min before adding the conjugates. Sampling was performed throughout the experiment, and sampled blood was immediately mixed with EDTA to the final concentration of 10 mM. The platelets were counted at 0, and at the end time point, Coulter AcT Diff Hematology Analyzer was used to ensure that coagulation had not occurred during the experimental procedure and as a response to the reagents added. Plasma was collected and stored at −80°C.

FACS analysis

Whole blood was stained with Abs purchased from BioLegend: anti-CD19 (Clone HIB19), anti-CD14 (Clone M5E2), anti-CD66b (Clone G10F5), CD1c (Clone L161), CD11c (Immunotech), CD123 (Clone 6H6), anti-CD16 (Clone 3G8), anti-CD32 (Clone 7.3), anti-CD64 (Clone 10.1), CD3 (clone UCHT1), CD4 (Clone OKT4), CD8 (Clone SK1), IFN-γ (Clone 4S.B3), and TNF-α (clone MAb11). For the detection of CMV-specific T cells, the iTAg Tetramer- HLA-A*0201 CMV PP65 (NLVPMVATV) from MBL International was used. A rabbit anti–Alexa Fluor 488 polyclonal Ab (Invitrogen) and trypan blue (Life Technologies) were used to quench the cell surface fluorescence. The RBCs were lysed using FACS lysing solution (BD Biosciences), the cells were analyzed using FACS Canto II (BD Biosciences), and the cell populations were gated using FlowJo (Tree Star).

Intracellular staining

The intracellular staining of IFN-γ and TNF-α was performed by adding Brefeldin A (Sigma-Aldrich) after 2 h of circulation of conjugates in the blood loop system. The experiment was terminated after another 4 h, as described for the blood loop system above. Surface markers were stained in whole blood prior to lysis of RBCs (BD Biosciences). The cells were washed once before permeabilization for 10 min at room temperature using Perm/Wash Buffer (BD Biosciences). The cells were stained for IFN-γ and TNF-α for 30 min at 4°C in the dark and subsequently washed in PBS with 1% BSA and 3 mM EDTA (Sigma-Aldrich).

Detection of circulating anti-OVA and anti-MTTE IgG Abs

The anti-OVA, antitetanus, and anti-MTTE IgG titers in donor plasma were evaluated with an in-house ELISA. Plates (3590; Corning) were coated with either OVA (5 μg/ml) (Sigma) or TTd (5 μg/ml) diluted in coating buffer (Carbonate-Bicarbonate Buffer; Sigma-Aldrich) and Streptavidin Plates (Thermo Fisher) were coated with MTTE-Biotin or ETTM-Biotin (1 μM) diluted in PBS overnight at 4°C. The plates were blocked with PBS (1% BSA and 0.05% Tween) and washed with PBS (0.05% Tween). The plasma samples were incubated on the plates for 2 h at room temperature. The circulating Abs were detected using a rabbit α-human IgG HRP–conjugated Ab (polyclonal; diluted 1:4000) and tetramethylbenzidine (Dako). The reaction was stopped with 1 M H_2SO_4, and the absorbance was read at 450–570 nm using an EMax Precision Microplate Reader (Molecular Devices). The results were analyzed using SoftMax version 2.35 (Molecular Devices). The detection of IgG isotypes and IgM were as described above with the following exceptions: the plates were blocked with PBS (10% BSA and 0.05% Tween), and the HRP-conjugated Abs were anti-IgG1 (Clone HP6070; diluted 1:500), anti-IgG2 (Clone HP6014; diluted 1:250), anti-IgG3 (Clone HP6047; diluted 1:250), and anti-IgG4 (Clone HP6023; diluted 1:500) from Thermo Fisher and anti-IgM from Dako (polyclonal; diluted 1:1000).

Ethical considerations

Blood sampling and diphtheria, tetanus, and pertussis (DTP) vaccination of healthy volunteers was approved by the local ethical committee. In short, an 18G gauge needle attached to heparinized tubing was used to draw blood. The blood was collected in a 50 ml surface heparinized tube and subsequently transferred to the loop tubing and then set to rotate as described above.

The DTP vaccination was performed by routine personnel at the hospital using a standard vaccine mixture.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.02 software (GraphPad software). Statistical analysis was calculated with

either Friedman test with Dunn correction or the Wilcoxon signed-rank test: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

Results

The tetanus-epitope targeting approach is superior to naked peptides in boosting recall responses of NLV- and GIL-specific CD8$^+$ T cells in human whole blood

With the aim to use Ag-specific Abs to improve targeting of tumor Ags to DCs, a peptide conjugate was constructed, linking an SLP with a B cell epitope. The B cell epitope is a peptide derived from the tetanus toxin protein. To evaluate the interaction of the conjugate with circulating antitetanus Abs where also complement is active, an ex vivo whole blood loop assay was used. In the loop assay, freshly isolated blood from healthy donors is set to rotate in loop-formed surface heparinized polyvinyl chloride tubings to mimic natural blood circulation. To establish if the tetanus epitope (referred to as MTTE) can facilitate T cell priming of a selected epitope, a conjugate (referred to as $[MTTE]_3$-NLV) consisting of an SLP containing a CMV-derived CD8 epitope (NLVPMVATV) (Fig. 1A, Table I) was synthesized. The $[MTTE]_3$-NLV conjugate induced IFN-γ and TNF-α recall responses of NLV-specific CD8$^+$ T cells in blood from donors that were both CMV and HLA-A*0201 positive (Fig. 1B). When comparing the naked peptide and the conjugate, it is possible to achieve similar responses with naked peptides, but this requires around 100-fold more peptide than conjugate (Fig. 1C). The $[MTTE]_3$-NLV conjugate also contains a CD4$^+$ epitope (ARNLVPMVATV) (20); however, no recall responses were seen in CD4$^+$ T cells, most likely explained by the donors' HLA-DRB5–negative profile (data not shown). To determine if the MTTE sequences can enhance T cell responses to alternative epitopes than the NLV epitope, we made MTTE conjugates with an influenza-derived CD8$^+$ epitope (GILGFVFTL) in a longer peptide stretch (See Table I). These conjugates also induced IFN-γ and TNF-α recall responses of GIL-specific CD8$^+$ T cells in blood from an HLA-A*0201–positive donor (Fig. 1D).

The conjugation of at least three MTTE sequences to an SLP is required to boost IFN-γ recall responses of NLV-specific CD8$^+$ T cells in human whole blood

ICs can have an adjuvant effect and aid T cell priming regardless if conjugated to an Ag or not; we therefore investigated if the recall responses induced by $[MTTE]_3$-NLV relied on the conjugation of the Ab targeting sequence ($[MTTE]_3$) or if the $[MTTE]_3$ and SLP induced T cell recall responses as separate entities. The conjugation of the SLP (NLV) with the $[MTTE]_3$ epitope was required for boosting an IFN-γ recall response, as the response was absent when incubating the two unconjugated entities in the same sample (Fig. 2A). In addition, the conjugation of three MTTE sequences was superior in T cell activation, as conjugates carrying one or two MTTE per SLP did not induce as high NLV-specific IFN-γ release by T cells (Fig. 2B). To determine if the $[MTTE]_3$-NLV conjugate requires circulating human anti-MTTE IgG Abs to induce an IFN-γ recall response, the conjugate was titrated and compared with a control substance ($[ETTM]_3$-NLV). The control substance has a scrambled MTTE peptide sequence (referred to as ETTM), which eliminates the anti-MTTE Ab binding site, and donors do not have IgG Abs detectable to this sequence (determined by ELISA, data not shown). The $[MTTE]_3$-NLV conjugate, but not the $[ETTM]_3$-NLV conjugate, boosted recall responses of NLV-specific CD8$^+$ T cells in a concentration-dependent manner (Fig. 2C). In agreement, the importance of the MTTE sequence was evident by the fact that an IFN-γ response was reduced in a donor recently vaccinated with DTP when preincubating the blood with a "cold" conjugate without the SLP (NLV) sequence ($[MTTE]_3$) but not with a cold $[ETTM]_3$ conjugate, in which the aim of the preincubation was to consume circulating anti-MTTE IgG Abs (Fig. 2D). Supportively, a mouse anti-MTTE IgG2a Ab could enhance a weak IFN-γ recall response induced by $[MTTE]_3$-NLV in a donor that had not recently been vaccinated (Fig. 2E).

The tetanus-epitope targeting strategy improves Ag uptake into human blood monocytes and CD1c$^+$ DCs in an Ab-dependent manner

As the NLV recall response is enhanced when conjugating the MTTE sequences to the SLP (NLV) sequence, we next analyzed which blood immune cells take up the conjugates. This was performed by conjugating the three MTTE sequences to an Alexa Fluor 488–labeled peptide, a conjugate hereafter named $[MTTE]_3$-AF488. As illustrated in Fig. 3A, $[MTTE]_3$-AF488 bound to blood monocytes, and this binding was enhanced in some donors by preincubating the conjugate with raMTTE IgG Abs (preformed ICs). Preformed ICs, but not $[MTTE]_3$-AF488 alone, bound B cells (Fig. 3A). In contrast, Ab-complexed $[MTTE]_3$-AF488 conjugates did not bind granulocytes (data not shown). No conjugate interaction was seen on FcγR-negative cells like T cells (data not shown). The data resemble the uptake pattern of the model Ag OVA when using Alexa Fluor 488–labeled OVA alone or as preformed complexes using raOVA IgG, with the difference of additional uptake by granulocytes (Supplemental Fig. 1A). By quenching the surface-bound $[MTTE]_3$-AF488 with an anti–Alexa Fluor 488 Ab, the cellular localization (extra- or intracellular) of the conjugate was determined. $[MTTE]_3$–AF488 complexes (with both circulating and rabbit Abs) were internalized by monocytes, whereas B cells partially internalized and partially adhered to rabbit Ab–complexed $[MTTE]_3$-AF488 conjugates (Fig. 3B), resembling the uptake pattern of OVA (Supplemental Fig. 1A). The fluorescent signal by monocytes was maintained over time, whereas the signal by B cells was lost over time (Fig. 3B), suggesting that B cells initially bind complexes without actively internalizing them, possibly acting as transporters of the conjugates. Blood monocytes also internalized the conjugate in the absence of raMTTE IgG Abs (Fig. 3A, 3B). To determine if this uptake was dependent on circulating human anti-MTTE IgG Abs, the conjugate was titrated and compared with the control conjugate $[ETTM]_3$-AF488, to which the donors lack IgG Abs against (data not shown). Monocytes specifically internalized the $[MTTE]_3$-AF488 conjugate, but not the ($[ETTM]_3$-AF488 conjugate, illustrating the requirement of circulating anti-MTTE Abs (Fig. 3C, 3D). In agreement, the circulating human anti-MTTE IgG levels were reduced in plasma samples taken from executed loop experiments (seen in four out of nine donors) in the presence of $[MTTE]_3$-AF488 (data not shown); this was also noted when using OVA in the loop system (data not shown). Consistently, raMTTE IgG Abs enhanced monocyte uptake of $[MTTE]_3$-AF488 but not $[ETTM]_3$-AF488 (Supplemental Fig. 1B). The uptake of $[MTTE]_3$-AF488 was analyzed using monocytes, as they are abundant APCs in whole blood. To confirm that professional APCs also take up the conjugates, uptake by blood DCs was confirmed by staining for CD1c$^+$CD11c^{high}CD123^{low} and CD19$^-$ cells (Supplemental Fig. 2) (21). Blood DCs take up $[MTTE]_3$-AF488 in a similar manner to monocytes (Fig. 3E).

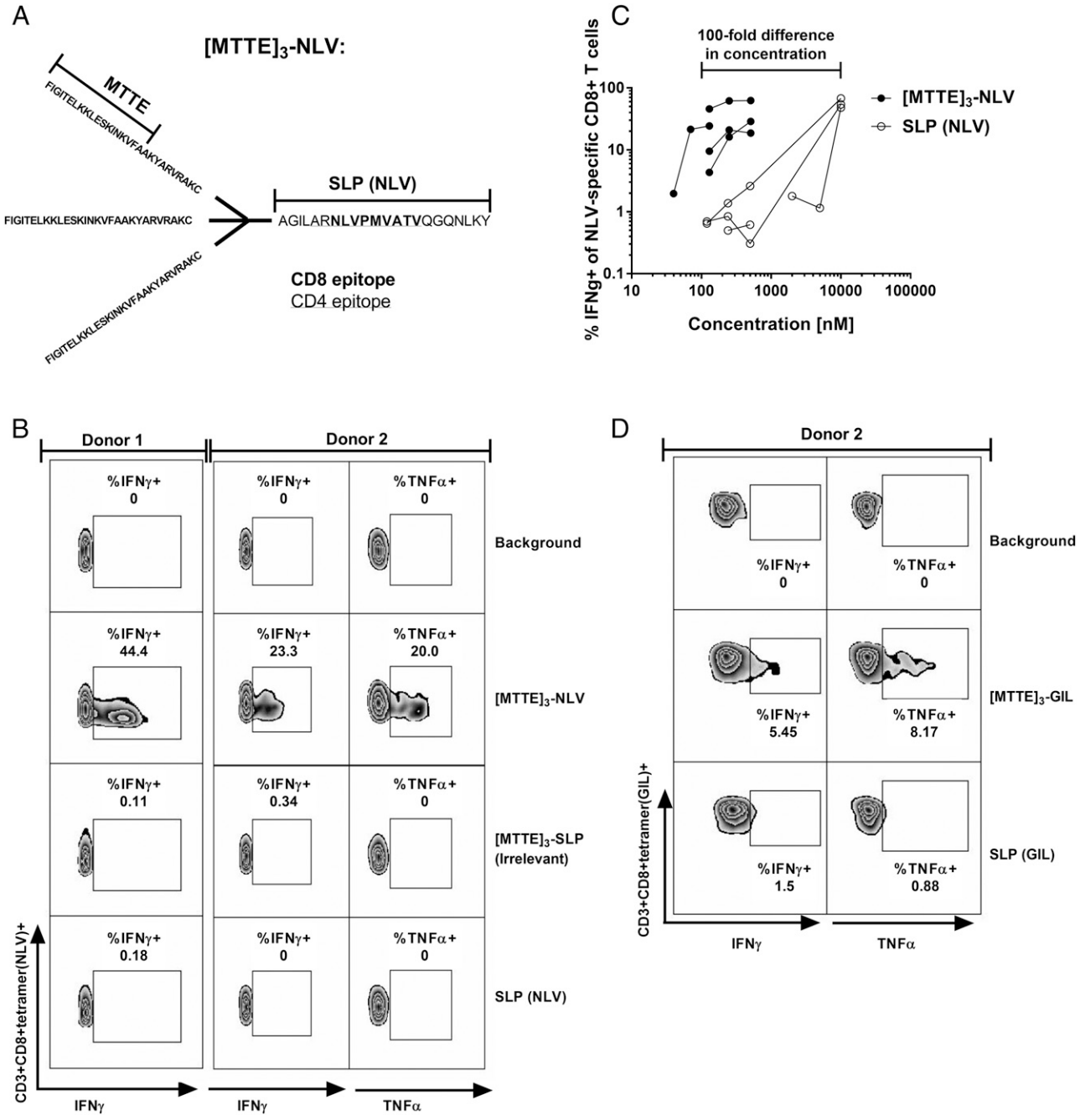

FIGURE 1. The peptide-conjugates with NLV- and GIL- $CD8^+$ epitopes boost IFN-γ and TNF-α recall responses of epitope-specific $CD8^+$ T cells in human whole blood. The peptide-conjugate $[MTTE]_3$-NLV (**A**), containing the CD8 epitope pp65(NLV) from CMV, was incubated at a final concentration of 120 nM in human whole blood from HLA-A*0201$^+$ and CMV^+ healthy donors in a circulating blood loop assay. After 2 h, Brefeldin A was added, and after a total of 6 h, aliquots were harvested and stained for T cell markers, tetramer HLA-A*0201 CMV pp65 (NLVPMVATV), and intracellular IFN-γ and TNF-α and analyzed with flow cytometry (**B**). The IFN-γ release in response to $[MTTE]_3$-NLV was compared with up to ~100-fold higher SLP (NLV) concentration [(**C**), $n = 3$]. The peptide-conjugate $[MTTE]_3$-GIL (480 nM) containing the CD8 epitope GIL from influenza (GILGFVFTL) was run as described above [(**D**), $n = 1$].

$[MTTE]_3$-AF488 uptake by monocytes and IFN-γ recall responses of NLV-specific $CD8^+$ T cells are partly dependent on the classical complement pathway

The uptake mechanism of $[MTTE]_3$-SLPs by monocytes was investigated by blocking FcγR, which are known to facilitate uptake of Ag–Ab complexes. By individual blocking of CD16, CD32, and CD64, the uptake of $[MTTE]_3$-AF488 by monocytes was not affected (data not shown). Furthermore, to eliminate any redundancy, CD16, CD32, and CD64, all expressed by blood monocytes (Supplemental Fig. 3), were all blocked at once, and as a consequence, monocytes slightly increased their uptake of the conjugates (data not shown). To determine the route of uptake, the complement system was blocked with the chelating agents EDTA and EGTA. EGTA blocks the classical pathway (CP), whereas EDTA blocks both the classical and the alternative pathway (AP) (22, 23). The uptake of $[MTTE]_3$-AF488 by monocytes was abolished by both EDTA and EGTA addition, but not by specifically blocking the AP with the C3-specific inhibitor Compstatin, indicating a role of the CP in the uptake mechanism (Fig. 4A). Consistently, EDTA and EGTA, but not Compstatin, reduced the percentage of IFN-γ–producing NLV-specific $CD8^+$ T cells in response to $[MTTE]_3$-NLV (Fig. 4B). In the mouse, C1q of the CP is crucial for the presentation

Table I. Peptide sequences

Abbreviations	Sequence
$[MTTE]_3$-NLV	[FIGITELKKLESKINKVF-AAKYARVRAKC]$_3$ - CORE LINKER- SLP (NLV)[a]
$[ETTM]_3$-NLV	[EKLINKLSKIFKGTIEVF-AAKYARVRAKC]$_3$ - CORE LINKER - SLP (NLV)
$[MTTE]_3$-AF488	[MTTE]$_3$ - CORE LINKER - AFKSKACB-Alexa Fluor 488
MTTE-Biotin	FIGITELKKLESKINKVF-SSSAFADVEAAZO-Biotin[b]
SLP (Irrelevant)	DGLQGLLLGLRQRIETLEGK
SLP (NLV)	AGILAR**NLVPMVATV**QGQNLKY[c]
SLP (GIL)	DLEALMEWLKTRPILSPLTK**GILGFVFTL**TVP[c]
C1q binding peptide	CEGPFGPRHDLTFCW
Control peptide	FPLRAPTFFVRRTIG

[a]The underlined MTTE sequence and the core linker are described in Ref. 17.
[b]O Lys(biotin).
[c]CD4 epitopes are underlined, and CD8 epitopes are bolded.
B, amide; Z, aminohexane acid.

of complexed OVA (24) also in cross-presenting DCs (25), and the uptake of complexed OVA by human monocytes is C1q-dependent in human whole blood (Supplemental Fig. 4). To determine if C1q is involved in the uptake of $[MTTE]_3$-AF488 by human monocytes, blood was preincubated with a C1q binding peptide with the aim to block C1q activity. The uptake of $[MTTE]_3$-AF488 was reduced when blocking the CP component C1q (Fig. 4C). Interestingly, the C1q binding peptide block completely abolished the IFN-γ–producing NLV-specific $CD8^+$ T cells in response to $[MTTE]_3$-NLV (Fig. 4D). Additionally, an anti-C1q–blocking Ab also blocked the uptake of $[MTTE]_3$-AF488 by monocytes and reduced the percentage of IFN-γ–producing NLV-specific $CD8^+$ T cells in response to $[MTTE]_3$-NLV in some donor blood (data not shown).

The analyzed blood donors have circulating IgG Abs against the MTTE peptide

As the conjugate uptake by monocytes and the induction of recall responses is dependent on circulating Abs, the IgG versus IgM profile was investigated in an in-house ELISA. The setup was assessed using the IgG preparation of TetaQuin (Sanquin, Netherlands; isolated and pooled IgG preparation from donors with established high titers against TTd) and all four IgG isotypes, IgG1–4, were detected against TTd (Fig. 5A). In plasma, from a randomly selected donor pool that had not recently been vaccinated with DTP, MTTE-specific IgG, but not IgM, Abs were detected (Fig. 5B). The OD values varied greatly between donors, but in all donors, the IgG OD values were above a cutoff of 1.1 of MTTE/ETTM fold change, whereas there was no positive score for the IgM OD value with this selection criteria. Circulating antitetanus IgG1 and IgG4, but not IgG2 and IgG3, Abs were detected (data not shown). This is consistent with published data of IgG isotype profiling post-DTP vaccination (26). Because the induction of T cell recall responses was found Ab-dependent (Fig. 2), the next step was to evaluate if a tetanus booster vaccination could enhance MTTE-specific IgG titers and conjugate induced T cell responses against the linked T cell epitope.

A tetanus vaccination boosts MTTE-specific IgG1 levels and enhances IFN-γ recall responses of NLV-specific $CD8^+$ T cells

Although the recall responses induced by $[MTTE]_3$-NLV can be enhanced by mouse anti-MTTE Abs, this approach would require an additional clinical development of a human IgG with a MTTE specificity to the current vaccine program. Therefore, a tetanus vaccination booster was investigated as a way to increase circulating anti-MTTE Abs, with the ultimate aim to enhance the recall response. The vaccination enhanced anti-MTTE IgG titers (median 1:2400 before and 1:12,800 after vaccination), but not IgM titers, in healthy volunteers (Fig. 6A, Table II). Furthermore, the vaccination enhanced the titers of anti-MTTE IgG1 Abs (median 1:10 before and 1:100 after vaccination) in all donors and IgG4 in one out of six donors, whereas IgG2 and IgG3 were undetected (Fig. 6B, Table II). Additionally, the vaccination greatly enhanced the recall responses induced by $[MTTE]_3$-NLV in blood from the two donors that were $HLA\text{-}A2^+CMV^+$ (Fig. 6C). Before donors were given the DTP vaccination, an addition of mouse anti-MTTE IgG2a into the loops enhanced the responses (Fig. 2E), whereas after the DTP vaccination, IgG2a and IgG1 decreased the responses (data not shown).

Discussion

T cells and their role in the control of viral diseases and malignancies are well recognized. Novel cancer therapies, such as checkpoint inhibitors, rely on the tumor to release Ags and the checkpoint inhibitors to release the break on these activated cells. For many patients, it is also of importance to boost both frequencies of tumor-reactive T cells and their activation through delivery of Ag material. Many existing therapeutic vaccines are hampered by poor Ag delivery, tolerance induction, Ag competition, and HLA limitations, among other pitfalls (2, 15, 27). In this study, we have focused on improving SLPs by IC formation, which is one of the most efficient pathways of delivering Ags and activating T cells. Key publications demonstrate both the efficiency of complexes to load APCs with Ag and the adjuvant capacity through stimulation of activating FcγR (8, 9, 13). Diseases linked to ICs (28, 29) and the T cell–mediated tissue destruction also indicate the potential of the strategy and the importance of choosing the right viral/tumor Ags to be combined with the strategy.

We have previously identified an 18-mer B cell epitope (MTTE) from tetanus toxin (17). For the current study, we use conjugates of MTTE attached to an Alexa Fluor 488–labeled peptide or a model T cell epitope to study cellular distribution and the mode of action of the vaccine strategy in a human whole blood assay. The interaction of Ag–Ab complexes with human whole blood was analyzed using an ex vivo blood loop assay with intact complement cascade systems. Both the model Ag OVA and our Alexa Fluor 488–labeled conjugate ($[MTTE]_3$-AF488) rapidly bound to monocytes and B cells in blood. Outer surface quenching of the fluorophore indicates that the Ag was internalized in monocytes, whereas B cells acted more to retain Ag in circulation without ingesting it. Preformed complexes could improve uptake in most, but not all, donors of both model systems. As B cells only express

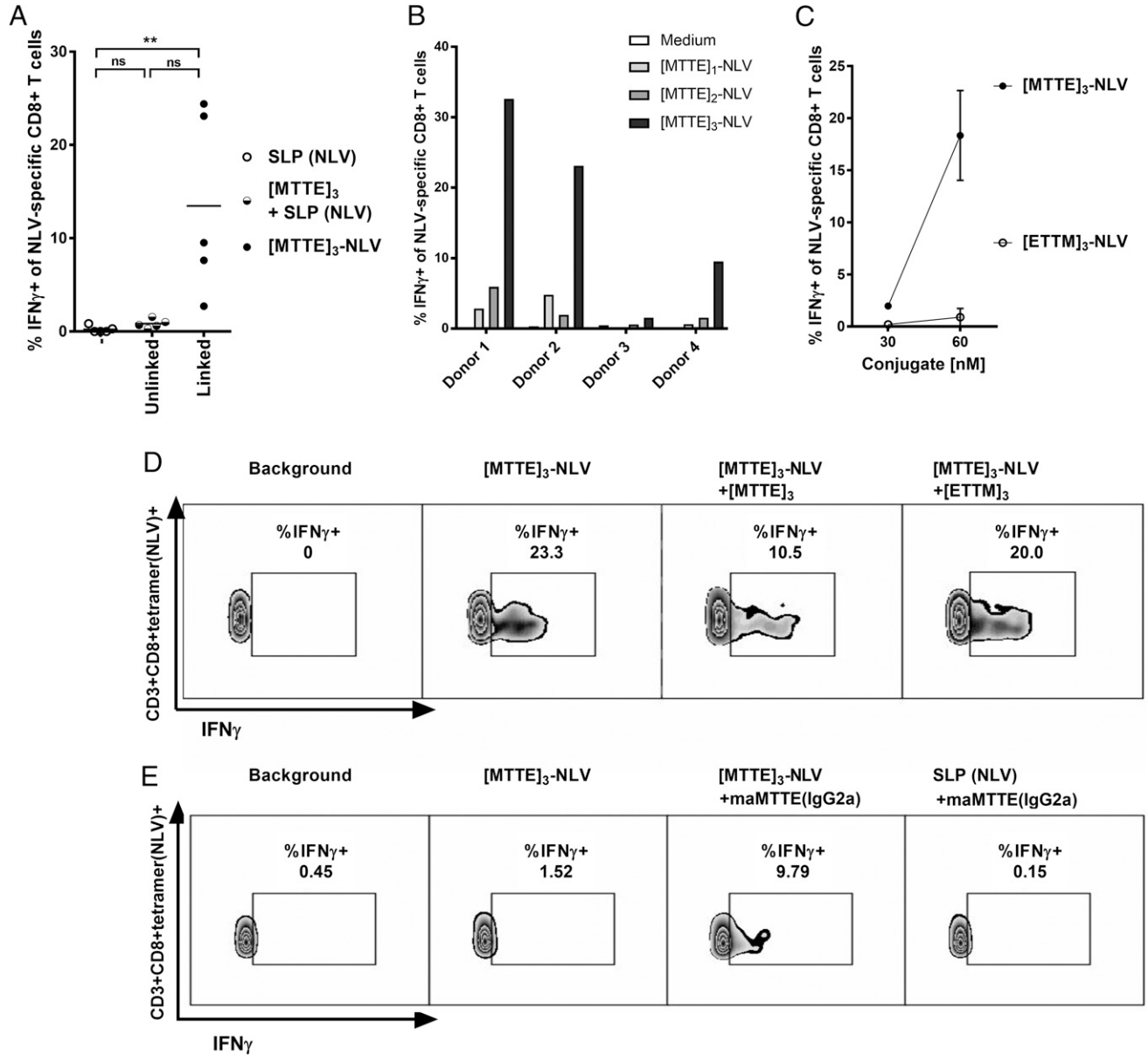

FIGURE 2. The conjugation of at least three MTTE sequences per SLP is required to boost IFN-γ recall responses of Ag-specific $CD8^+$ T cells in human whole blood. The peptide-conjugate $[MTTE]_3$-NLV (Fig. 1A) was incubated at a final concentration of 120 nM in whole blood from HLA-A*0201$^+$ and CMV^+ healthy donors in a circulating blood loop assay. After 2 h, Brefeldin A was added, and after a total of 6 h, cells were stained for tetramer HLA-A*0201 CMV pp65 (NLVPMVATV) and intracellular IFN-γ and analyzed with flow cytometry. $[MTTE]_3$-NLV in a conjugated form was compared with the two separate entities ($[MTTE]_3$ + SLP [NLV]) [(**A**), $n = 5$ donors run at separate occasions, and the lines represent the mean]. IFN-γ responses induced by conjugates with one, two, or three MTTE sequences were compared, and the percentage of IFN-γ^+ NLV–specific cells induced by conjugates with one, two, or three MTTE is shown [(**B**), $n = 4$ donors run at separate occasions]. IFN-γ responses induced by $[MTTE]_3$-NLV were compared with that of a conjugate containing a scrambled MTTE sequence $[ETTM]_3$-NLV, showing the mean and SD [(**C**), $n = 2$ donors run at separate occasions]. The cold conjugate without an SLP ($[MTTE]_3$ or the scrambled control conjugate $[ETTM]_3$) was preincubated in the blood (from a donor that mounts a strong T cell response against $[MTTE]_3$-NLV) for 10 min before $[MTTE]_3$-NLV was added and analyzed as described above [(**D**), $n = 1$]. The IFN-γ response of a donor that mounts a poor T cell response against $[MTTE]_3$-NLV, was analyzed with the addition of a mouse anti-MTTE IgG2a Ab at the final concentration of 40 μg/ml [(**E**), one representative donor out of three]. The data were analyzed with Friedman test with Dunn correction. $^{**}p < 0.01$.

the inhibitory CD32b, whereas monocytes express CD32a/c or CD16a and/or complement receptors, Ag uptake is likely mediated by these activating low-affinity FcR or innate receptors. The uptake of fluorescent $[MTTE]_3$-AF488 was analyzed using human monocytes as an abundant type of APCs in whole blood. To confirm uptake by professional APCs, whole blood was stained for DCs ($CD11^{high}CD123^{low}CD1c^+$ and $CD19^-$) (21), which were found able to take up $[MTTE]_3$-AF488. Importantly, uptake by DCs was similar to monocytes, suggesting that this in vivo DC-loading technology can be useful for many types of Ags.

The prospect of the conjugate as a vaccination strategy relies on its ability to prime or activate SLP-specific T cells. To study T cell activation, we made use of a recall assay using CMV as a model Ag. Blood from HLA-A*0201$^+$ and CMV-positive donors was used to assess how cytotoxic T cells respond to our model conjugate ($[MTTE]_3$-NLV) as compared with control conjugates. Both IFN-γ and TNF-α responses were induced by NLV-specific T cells in response to our novel peptide conjugate (after only 6 h of incubation), whereas control conjugates failed to induce T cell activation. Aggregated data of various concentrations of conjugates

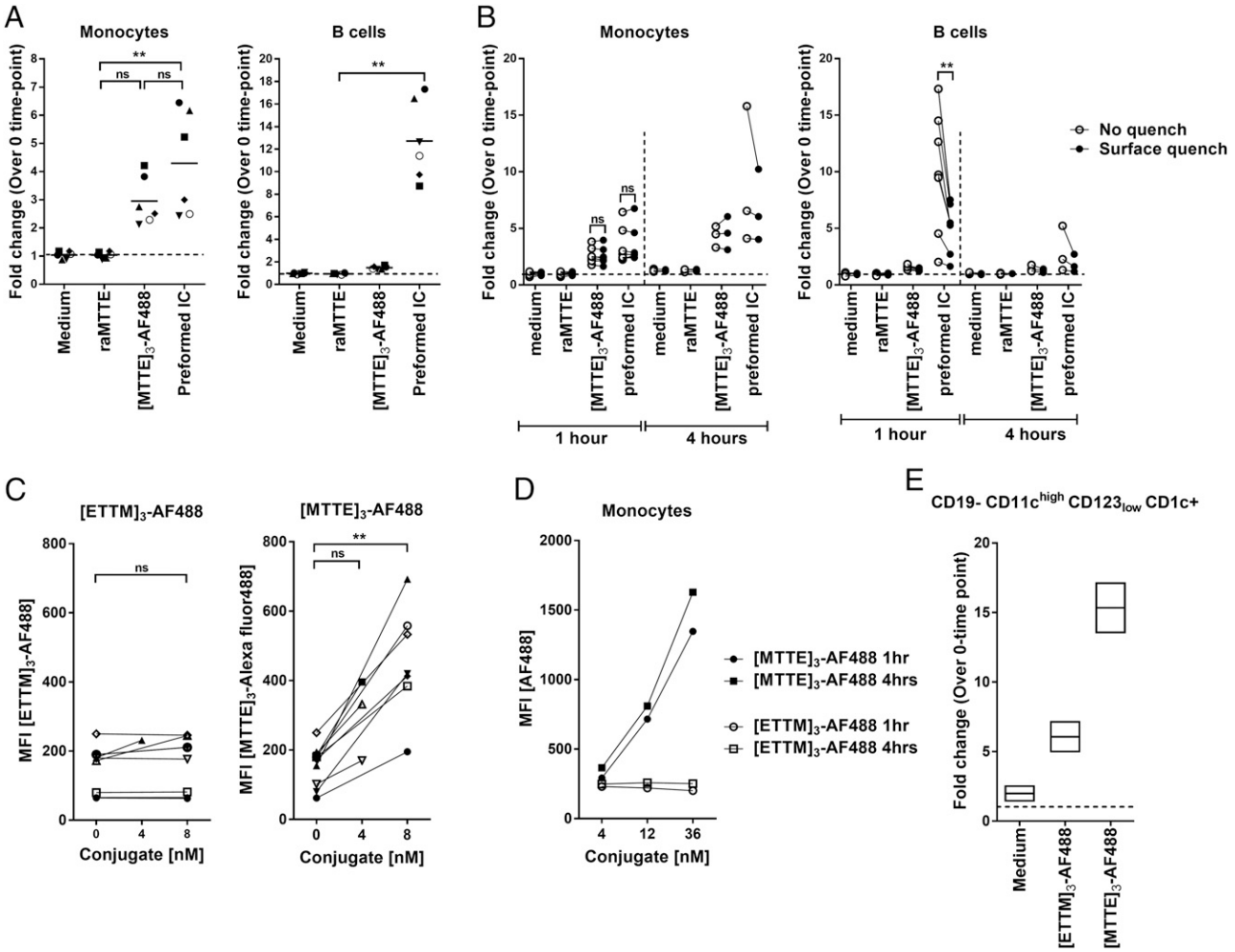

FIGURE 3. The uptake of $[MTTE]_3$-AF488 by human blood monocytes and B cells is Ab dependent. Alexa Fluor 488–labeled $[MTTE]_3$-SLP, ($[MTTE]_3$-AF488), raMTTE, or in vitro preincubated $[MTTE]_3$-AF488 and raMTTE (preformed IC) were incubated in blood from healthy donors in a circulating blood loop assay. After 1 h (and 4 h where indicated), aliquots were harvested and stained for monocytes ($CD14^+CD19^-$) and B cells ($CD19^+$ $CD14^-$). The uptake was analyzed with flow cytometry, and the fold change in mean fluorescence intensity (AF488) postloop/preloop (0 time point) is presented in [(**A**), $n = 6$]. The surface-bound AF488 (1 and 4 h) was quenched with an anti–Alexa Fluor 488 Ab [(**B**), 1 h, $n = 8$ and 4 h, $n = 3$]. The uptake of $[MTTE]_3$-AF488 and $[ETTM]_3$-AF488 by monocytes [(**C**), $n = 9$] and by blood-derived DCs [(**E**), $n = 2$]. The uptake pattern by monocytes of $[MTTE]_3$-AF488 and $[ETTM]_3$-AF488 in a titration [(**D**), $n = 1$]. The data were analyzed with the Friedman test with Dunn correction (A) or the Wilcoxon signed-rank test (B and C). The lines represent the mean. $^{**}p < 0.01$.

versus SLPs alone demonstrate that approximately a 100-fold less conjugate (equimolar range) was required to induce similar IFN-γ responses, which may reflect both the increase in active Ag uptake along with an optimized intracellular processing pathway for cross-presentation induced by ICs (9, 16). The more potent recall responses associated with the MTTE-linked T cell epitopes were demonstrated to be not only applicable to the CMV epitope, as a conjugate with the influenza-derived epitope could boost IFN-γ release of influenza-specific T cells.

The tetanus-derived 18-mer peptide (MTTE) most likely creates a scenario in which the Abs are restricted to bind a single antigenic determinant, similar to mAbs; we therefore investigated whether one, two, or three MTTE sequences were optimal for T cell activation. It was clear that at least three B cell epitopes were required for T cell activation to occur, most likely due to formation of a complex rather than merely a monomeric Ab–carrying Ag, which should not have the capacity to trigger FcγR activation or trigger complement activation. Based on previous data, both Ag-loading and adjuvant effects through Fcγ/innate receptor stimulation are of importance in providing T cell priming/activation (8, 9). To test if the T cell activation induced by $[MTTE]_3$-NLV was only a result of the adjuvant part, the complexes and the T cell Ag were provided as a one-plus-one combination rather than one entity. This did not provide the same magnitude of T cell responses, demonstrating the need for linked adjuvant/Ag for optimal delivery/activation. Conjugation of the adjuvant and the Ag is most likely necessary for the improved loading of the Ag into the APCs and thereby facilitating enhanced T cell activation. In a setting where de novo priming plays a greater role than in recall settings, a role for the adjuvant (Fc stimulatory effect) can also be of importance. Additionally, conjugation of adjuvant and Ag is of importance for ensuring Ag uptake and activation of the same APC, something that is not ensured when long peptides are provided in, for example, mineral oil depots.

Whole anti-TTd isotype profiling has shown that IgG1 and IgG4 are prevalent isotypes toward the protein. A tetanus vaccine boost increased the donors' IgG titers, without the induction of IgM, against the MTTE epitope and promoted an increase in T cell activation in response to the $[MTTE]_3$-NLV model conjugate. The MTTE epitope has a partial overlap with a universal CD4

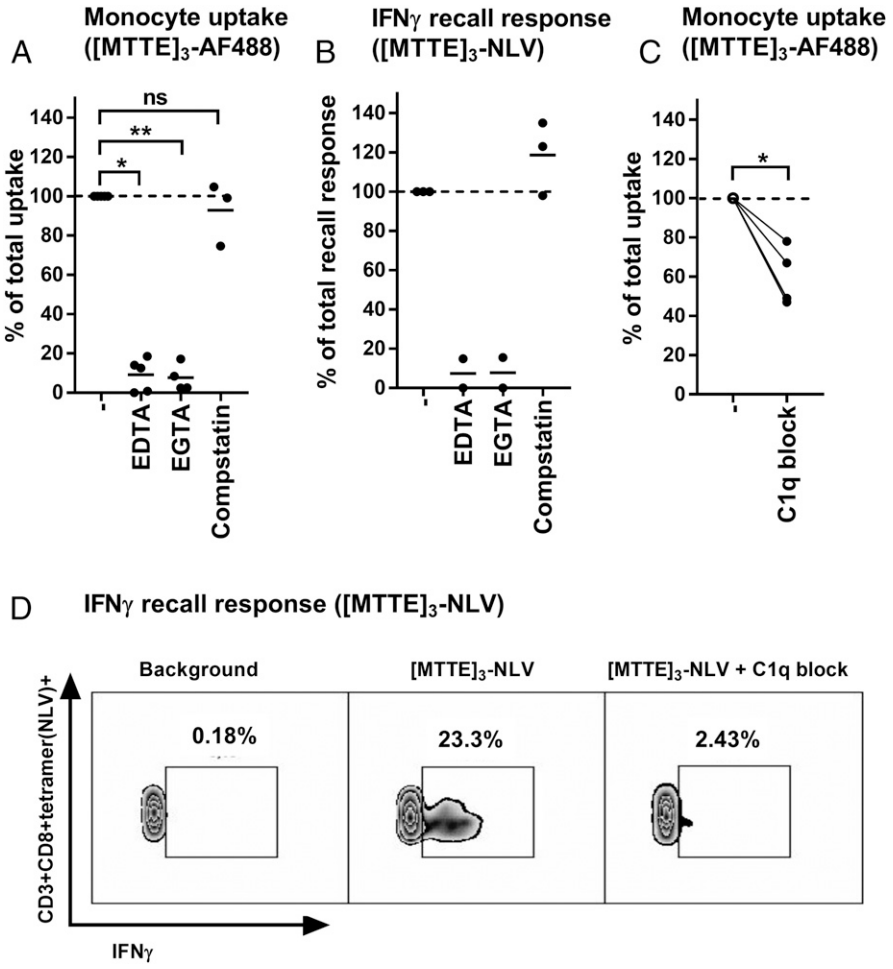

FIGURE 4. Blocking C1q reduces the uptake of $[MTTE]_3$-AF488 by monocytes and affects the IFN-γ recall responses boosted by $[MTTE]_3$-NLV. The uptake of Alexa Fluor 488–labeled $[MTTE]_3$-SLP ($[MTTE]_3$-AF488) by monocytes was blocked with EDTA ($n = 5$), EGTA ($n = 4$), Compstatin ($n = 3$) (**A**), or a C1q-blocking peptide ($n = 4$) (**C**). The blocking agents were preincubated for 10 min before the addition of $[MTTE]_3$-AF488 to blood from healthy donors in a circulating blood loop assay. After 1 h of incubation, aliquots were harvested and stained for blood monocytes ($CD14^+$ and $CD19^-$). The percentage of total uptake was calculated by subtracting the background (medium) mean fluorescence intensity (MFI) (AF488) followed by (MFI of block/MFI of $[MTTE]_3$-AF488) × 100 (A and C). The blocking agents were preincubated for 10 min before $[MTTE]_3$-NLV was added to whole blood from HLA-A*0201$^+$ and CMV$^+$ healthy donors. After 2 h, Brefeldin A was added, and after a total of 6 h, aliquots were harvested and stained for T cell markers, tetramer HLA-A*0201 CMV pp65 (NLVPMVATV), and intracellular IFN-γ and analyzed with flow cytometry. The percentage of total recall response was calculated by dividing (percentage of IFN-γ of block with percentage of IFN-γ of $[MTTE]_3$-NLV) × 100 [(**B**) and (**D**), $n = 1$]. The data were analyzed with the Wilcoxon signed-rank test. The lines represent the mean. $*p < 0.05$, $**p < 0.01$.

epitope QYIKANSKFIGITEL (30). It remains to be elucidated if the reason for the universal anti-MTTE response is because the T cell epitope is in proximity of the B cell epitope in the native protein.

In a clinical setting, patients might benefit from a tetanus vaccination prior to the initiation of the therapeutic conjugate vaccine cycle, and as tetanus vaccination can have beneficial effects on DC infiltration and activation at the vaccination site (31), there is an opportunity for mutual benefits by smart clinical trial design.

The Ab dependence of the conjugate was further confirmed by the selective Ag uptake and T cell activation of conjugates with the MTTE sequence and not the scrambled peptide (ETTM), which the donors lack Abs against. In an attempt to study the route of uptake, both FcγR and complement inhibitors were used. Whole IgG-blocking Abs against FcγR and C1q turned out to be difficult to

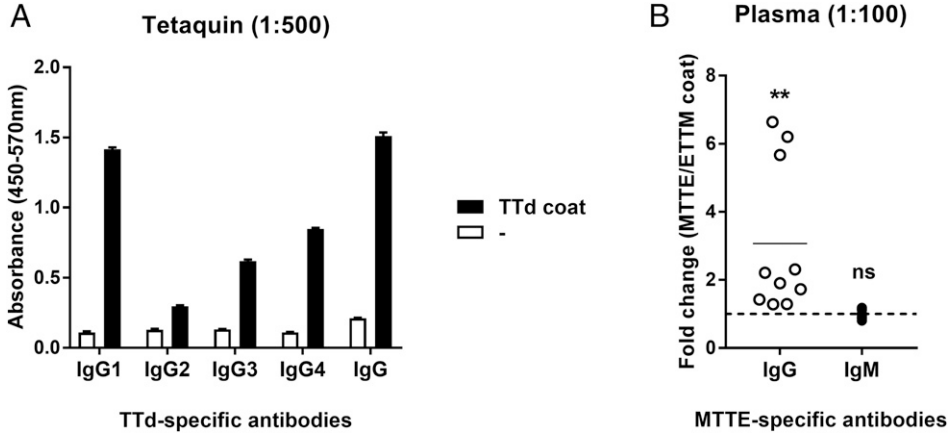

FIGURE 5. Healthy individuals have circulating IgG, but not IgM, Abs against the MTTE peptide. Antitetanus IgG titers of TetaQuin were determined by an in-house ELISA using plates coated with TTd and the detection Abs: polyclonal anti-human IgG and isotype-specific HRP-conjugated Abs [(**A**), repeated two times with similar results]. The presence of anti-MTTE IgG and IgM in plasma from blood loop donors was determined by coating streptavidin plates with either MTTE-biotin or ETTM-biotin, and the IgG Abs were detected by a polyclonal anti-human IgG HRP-conjugated Ab [(**B**), $n = 10$] or anti-human IgM HRP-conjugated Ab [(B), $n = 9$]. The data were analyzed with the Wilcoxon signed-rank test. The lines represent the mean. $**p < 0.01$.

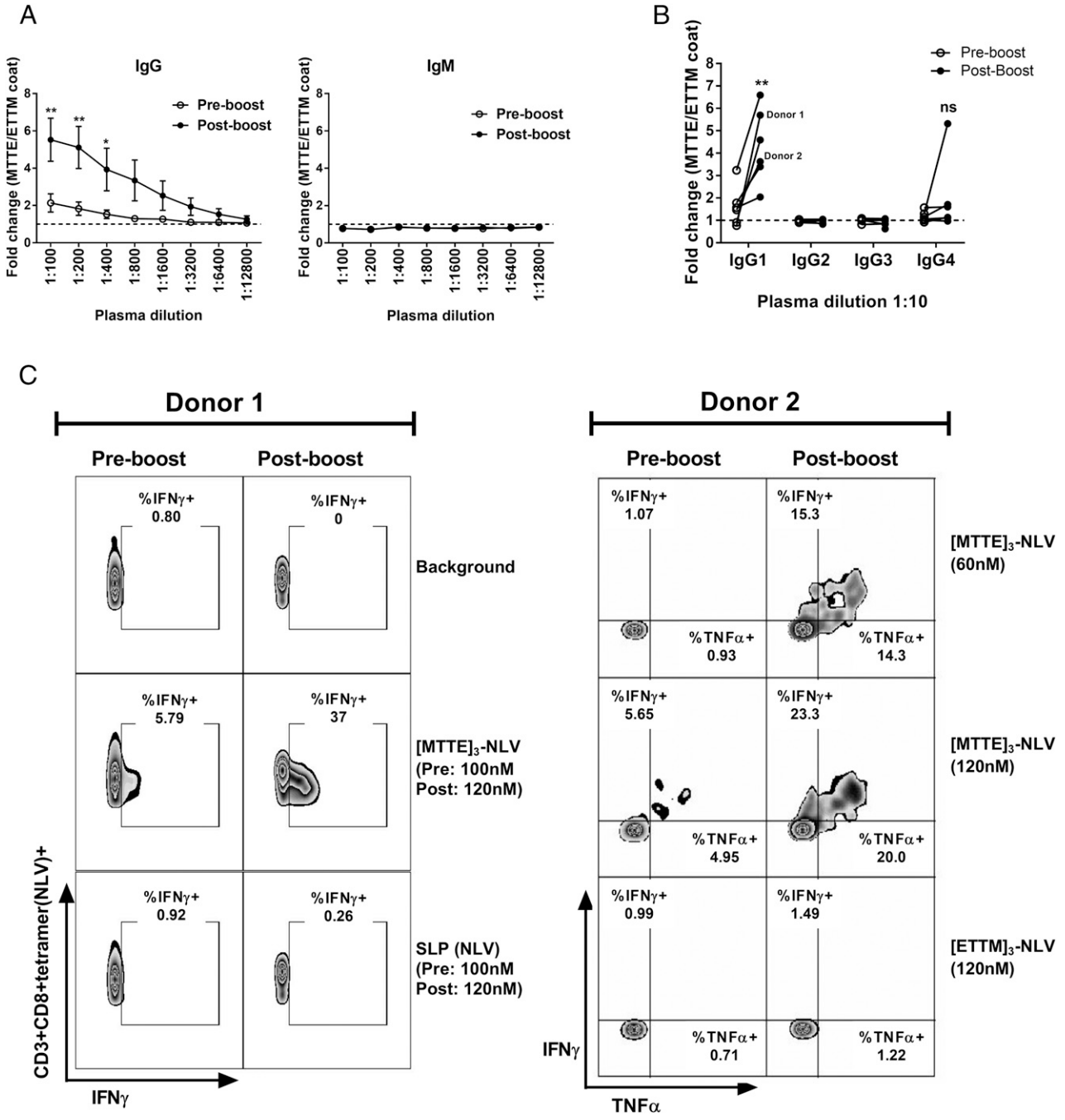

FIGURE 6. A standard tetanus booster vaccination elevates anti-MTTE IgG1 levels in plasma and enhances IFN-γ recall responses of NLV-specific $CD8^+$ T cells in response to $[MTTE]_3$-NLV. The anti-MTTE IgG and IgM levels in plasma pre- and posttetanus booster vaccination were determined in an in-house ELISA where streptavidin plates were coated with MTTE-biotin (or ETTM-biotin) and the Abs were detected with a polyclonal anti-IgG, polyclonal anti-IgM, or IgG isotype-specific HRP-conjugated Abs [(**A**), mean, $n = 6$ and (**B**), $n = 6$]. The peptide-conjugate $[MTTE]_3$-NLV was incubated in whole blood from HLA-A*0201$^+$ and CMV^+ healthy donors (pre- and posttetanus booster vaccination) in a circulating blood loop assay. After 2 h, Brefeldin A was added, and after a total of 6 h, aliquots were harvested and stained for T cell markers, tetramer HLA-A*0201 CMV pp65 (NLVPMVATV), and intracellular IFN-γ and TNF-α and analyzed with flow cytometry [(**C**), $n = 2$]. The data were analyzed with a two-way ANOVA. $^*p < 0.05$, $^{**}p < 0.01$.

use in a system with intact complement due to their Ab-dependent cell–mediated cytotoxicity/complement-dependent cytotoxicity–promoting effect in the blood assay when the Abs cover circulating cells. By the use of alternative complement blockers, we could identify a role of the CP in conjugate uptake as both EDTA (blocks AP and CP) and EGTA (blocks CP) affected uptake (23). Additionally, EDTA and EGTA blocked the recall responses induced by the $[MTTE]_3$-NLV conjugate. However, EDTA and EGTA influence several Mg^{2+}- and Ca^{2+}-dependent cellular functions and therefore do not exclusively implicate the CP as the route of uptake. The CP component C1q was shown to be essential, rather than FcγR, for the uptake and cross-presentation of OVA–Ab complexes in mice (24, 25). In this study, the uptake of both OVA and $[MTTE]_3$-AF488 by human monocytes were found partly dependent on C1q rather than the C3-specific inhibitor Compstatin. Consistently, recall responses were blocked by the C1q inhibitor but not Compstatin. A role of C1q in conjugate uptake and induction of recall responses is consistent with its

Table II. Ab titers before and after DTP booster vaccination

Donor	Total IgG		IgG1		IgG4	
	Before	After	Before	After	Before	After
Donor 1[a]	1:3200	1:25600	ND	1:200	ND	1:10
Donor 2[a]	1:6400	1:25600	1:10	1:200	ND	1:10
Donor 3	1:1600	1:6400	1:10	1:10	1:10	1:10
Donor 4	1:100	1:12800	1:10	1:100	ND	ND
Donor 5	1:800	1:3200	1:10	1:100	1:50	1:200
Donor 6	1:25600	1:12800	1:50	1:100	1:50	1:50

The titers are expressed by the dilution whereby a fold change >1.1 is achieved of the ratio between the MTTE and the control-coated ETTM plate.
[a]The donors are represented in Fig. 6C.
ND, not detected.

ability to bind human IgG1 (32), which was the main donor anti-MTTE IgG isotype detected. Additionally, the enhanced recall responses by mouse anti-MTTE IgG2a can be explained by its ability to bind human C1q (33, 34). However, after the tetanus vaccination, mIgG2a and mIgG1 specific for the MTTE reduced the recall response, suggesting a competition with circulating human Abs of which the murine with higher affinity wins. The reduction of recall response could be a result of redirecting the uptake to other cell types or simply an effect on uptake via Fc receptors and/or complement fixation. Especially, mIgG1 is likely to block the uptake, considering its low affinity for both human FcγR (35) and C1q (33, 34); however, it is still unclear how mouse IgG2a can have a dual role in this. C1q expression, or presence, on the cell surface of monocytes (36, 37) can explain why blood monocytes, but not B cells or granulocytes, take up the conjugate. The maturation of monocyte-derived DCs (38) when cultured on immobilized C1q, which mimics C1q cross-linking upon IC binding, still supports this strategy as one with adjuvant potential, despite that FcγR does not appear involved in Ag uptake in human whole blood.

In summary, our strategy of targeting Ags via Ag–Ab complexes may improve T cell recall activation in humans if the responses mimic the ex vivo responses to CMV and influenza that are demonstrated in this study. As the blood loop assay measures recall T cell responses, it will also be of importance to study priming of naive T cells ahead. Clinically, most peptides are given as intradermal or i.m. injections; the optimal administration of our drug conjugate and the requirement of prevaccinated patients with tetanus vaccination remains to be investigated. Lastly, the data in this study supports the use of a selected B cell epitope as a carrier peptide for Ag material into DCs to improve Ag presentation and T cell activation.

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Disclosures

E.A.K.F. is the founder/co-owner of Immuneed AB and has stocks in Immuneed AB. S.M.M. is the founder/owner, Chief Scientific Officer, and a board member of Immuneed AB. Immuneed AB is developing a therapeutic cancer vaccine based on the technology presented. The other authors have no financial conflicts of interest.

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