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Id-neoantigen vaccine induces therapeutic CD8+ T cells against multiple myeloma: H chain-loss escapees cause FLC MM

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ABSTRACT

Background Multiple myeloma (MM) cancers originate from plasma cells that have passed through the germinal center reaction where somatic hypermutation of Ig V regions takes place. Myeloma protein V regions often express many mutations and are thus a rich source of neoantigens (traditionally called idiotopes (Id)). Therefore, these are highly tumor-specific and excellent targets for immunotherapy.

Methods We have developed a DNA Id vaccine which as translated protein targets conventional dendritic cells (cDC) for CCL3-mediated delivery of myeloma protein V regions in a single-chain fragment variable (scFv) format. Vaccine efficacy was studied in the mouse MM model, mineral oil-induced plasmacytoma 315.BM.

Results The Id vaccine protected mice against a challenge with MM cells. Moreover, the vaccine had a therapeutic effect. However, in some of the vaccinated mice, MM cells not producing H chains escaped rejection, resulting in free light chain (FLC) MM. Depletion of CD8+ T cells abrogated vaccine efficacy, and protection was observed to be dependent on cDC1s, using Batf3-/- mice. Modifications of scFv in the vaccine demonstrated that CD8+ T cells were specific for two mutated V_H sequences.

Conclusions V_H neoantigen-specific CD8+ T cells elicited by CCL3-containing Id vaccines had a therapeutic effect against MM in a mouse model. MM cells could escape rejection by losing expression of the H chain, thus giving rise to FLC MM.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Idiotypic (Id) vaccination of multiple myeloma (MM) patients has given disappointing results.

WHAT THIS STUDY ADDS

⇒ We have here developed a DNA Id vaccine that as translated protein targets single-chain fragment variable of a myeloma protein to dendritic cells via a CCL3-CCR interaction. Therapeutic vaccination of mice results in CD8+ T cells specific for two distinct neoantigenic sequences in the V_H of the preclinical MM model mineral oil-induced plasmacytoma 315. Variant MM cells lacking H chain expression escape rejection resulting in free light chain MM.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These results justify testing of CCL3-targeted Id vaccines in MM patients.

BACKGROUND

Tumor neoantigens recognized by T cells are important for tumor rejection and design of cancer vaccines.[1 2] Multiple myeloma (MM) cells arise from B cells that have undergone V(D)J gene rearrangements of Ig H and L chain genes during their development in the bone marrow.[3 4] Later, during the germinal center (GC) reaction,[5] the rearranged V(D)J is substrate for somatic hypermutation.[4 6] As a consequence of (1) V(D)J junctional diversity and (2) somatic hypermutation, the MM clone usually expresses unique Ig V regions with tumor-specific sequences that yield tumor specific peptides (neoepitopes) that can be presented by major histocompatibility complex (MHC) molecules. Such V region neoepitopes have traditionally been referred to as idiotypic (Id) peptides.

Id peptides are presented on MHC class II molecules to Id-specific CD4+ T cells[7 8] and MHC class I molecules to Id-specific CD8+ T cells.[9–11] Recently, Id peptides were eluted from a variety of human B cell malignancies.[12 13] Although there was a paucity of Id peptides eluted from MHC class I molecules,[12] Id-specific CD8+ T cells have been described in human MM.[11] Id vaccines may be clinically relevant since they prolonged disease-free survival in patients with follicular lymphoma.[14] A phase I study is under way for lymphoplasmacytic lymphoma.[15] We have

here explored the vaccine-induced Id-specific $CD8^+$ T cells in a preclinical model of MM.

We have previously developed a mouse model with features of human MM. In brief, from the mineral oil-induced plasmacytoma (MOPC)315,[16] we derived a cell line (MOPC315.BM) that could (1) be grown in vitro and (2) injected intravenous into immunocompetent BALB/c mice, resulting in development of a MM-like osteolytic disease in the bone marrow.[17]

The MOPC315.BM cells secrete a well-characterized IgAλ2 myeloma protein (M315) with four and eight missense mutations in the V_L[18] and the V_H regions,[19] respectively. Three of the expressed Vλ2 mutations are located in the third hypervariable region, constituting an Id-peptide that is presented on MHC class II molecules ($I\text{-}E^d$) to $CD4^+$ T cells.[8] Such Id-specific $CD4^+$ T cells have been shown in a TCR-transgenic model to mediate potent antitumor immune responses against the MOPC315 MM via induction of cytotoxic macrophages.[20] Protection against MOPC315 can also be elicited by prophylactic Id-vaccination. However, the mechanism of protection has never been fully established, although initial results suggested a contribution of $CD8^+$ T cells.[21]

Immunogenicity of vaccines can be enhanced by conjugating antigen to antibodies specific for antigen presenting cells (APC).[22 23] In a further development, plasmids encoding APC-specific proteins were used for DNA immunization.[21 24] As a means of targeting, chemokines specific for chemokine receptors, or single-chain fragment variable (scFv) of mAbs specific for cell surface molecules on APC, were used in these studies. The combination of APC-targeting and DNA delivery clearly enhanced T cell responses and protection against tumors.[21 24–26]

Here, we have used a DNA Id vaccine to elicit T cells that eradicate MOPC315.BM cells. The DNA vaccine encodes a bivalent chemokine-$scFv^{315}$ fusion protein that targets the Id of MOPC315 to dendritic cells (DCs). We found that such chemokine-enhanced Id vaccination induced V_H-specific $CD8^+$ T cells with a therapeutic effect against MOPC315.BM cells. The Id-specific $CD8^+$ T cells were elicited by two different neoantigens in the V_H 315 sequence. MOPC315.BM cells could escape $CD8^+$ T cells by abrogating their expressions of H chains, thus giving rise to free light chain (FLC) MM.

METHODS

Cell lines

The BALB/c MOPC315.4 cell line,[27] derived from an in vitro MOPC315 plasmacytoma cell line (ATCC, Manassas, Virginia, USA)[16] was repeatedly cycled between passages intravenous and in vitro culture to obtain a variant cell line that efficiently homed to bone marrow and generated osteolytic lesions (MOPC315.BM and the luciferase-labeled MOPC315.BM.Luc[17]). Human embryonic kidney (HEK) 293E cells were obtained from ATCC.

Cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, California, USA) supplemented with FCS (10%, heat-inactivated), non-essential amino acids (0.1 mM, Lonza, Allendale, New Jersey, USA), sodium pyruvate (1 mM, Lonza), monothiolglycerol (50 uM, Sigma) and gentamycin (24 mg/L, Sanofi-Aventis, Norway) at 37°C with 5% CO_2 in humidified air.

Mice

Female BALB/c mice aged 6–8 weeks old were purchased from Janvier, France. $BATF3^{-/-}$ mice on a BALB/c background were purchased from The Jackson Laboratory (Stock No.: 013755) and bred in-house. Mice were anesthetized with i.p. injection of a cocktail of Zoletil forte (250 mg/mL, Virbac, Carros, France), Rompun (20 mg/mL, Bayer Animal Health) and Fentanyl (50 ug/mL, Actavis, Parsippany-Troy Hills, New Jersey, USA) (ZRF) given at 6 mL/kg bodyweight, or isoflurane (Baxter Healthcare, Deerfield, Illinois, USA). Blood samples were collected from vena saphena or by cardiac puncture (at endpoint). Mice were euthanized by cervical dislocation.

Cloning of DNA vaccines

Vaccine constructs were expressed in the pLNOH2 vector containing the CMV promotor and the V_H-leader sequence derived from the V_H gene of B1-8 mAb.[28] The CCL3-$scFv^{315}$, CCL3-$scFv^{A20}$ and the $scFv^{aNIP}$-$scFv^{315}$ DNA vaccines have previously been described.[21] The various construct with V_H^{Germ},[19] Mut1-8 variants on the V_H^{Germ} and ΔMut1, ΔMut5, ΔMut6 and ΔMut5,6 on the V_H^{315}, all in a scFv with V_L^{315}, were purchased from Genscript and subcloned into the CCL3-$scFv^{315}$ DNA vaccines, thus exchanging the $scFv^{315}$ with the respective mutated versions. Correct sequences of the vaccine constructs were confirmed by sequencing (Eurofins). Vaccine plasmid DNA was purified with EndoFree Mega plasmid purification system (Qiagen).

DNA vaccination and electroporation

Mice were anesthetized with i.p. injection of ZRF. Each hind leg was shaved and sterilized before i.m. injection of plasmid DNA (25 µg in sterile 0.9% NaCl) into each quadriceps femoris.Electroporation (EP) was immediately performed through the delivery of pulses from inserted electrodes flanking the injection site (Needle EP), using an Elgen electroporator (Inovio Biomedical).[29]

Prophylactic vaccination and MOPC315 challenges: depletion of $CD8^+$ T cells

In the subcutaneous model,[27] mice vaccinated 14 days previously were challenged with MOPC315.4 cells (1×10^5) injected s.c. on the right flank. A tumor diameter of 15 mm was defined as a humane endpoint, at which time mice were euthanized. Blood samples were collected at day 13 after challenge for measurement of M315 myeloma protein.

In the bone marrow model of MOPC315, mice were challenged 14 days after immunization by intravenous injection of MOPC315.BM.Luc (2×10^5 cells/mouse),

MOPC315.BM.Luc.IgAλ2 (2×10^5 cells/mouse), or MOPC315.BM.Luc.λ2 (2×10^5 cells/mouse).[17] On development of paraplegia due to spinal compression, the mice were euthanized. Tumor load was followed by imaging using the IVIS Spectrum imaging system (Caliper Life Sciences, Hopkinton, Massachusetts, USA). Mice were injected with D-Luciferin (150 mg/kg body weight, i.p., Sigma Aldrich, St. Louis, Missouri, USA), anesthetized with isoflurane, and images acquired 10 min later. Data were analyzed using the LivingImage software (Caliper Life Sciences). For the CD8 depletion experiment, mice were immunized and challenged with MOPC315.BM.Luc cells as described above. Starting 12 days after immunization, mice received every other day injections of 100 µg depleting mAb (TIB-105; ATCC) for 9 days or with isotype control Ab (Y13-238, ATCC). For the remaining 4 weeks, mice received depleting antibodies once a week. In addition to imaging, tumor load was estimated by measurements of myeloma protein M315 in sera obtained on day 21, day 35 and at endpoint.

Therapeutic vaccination of MOPC315.BM-injected mice

Mice were challenged with MOPC315.BM.Luc.IgAλ2 (5×10^4 cells, intravenous) and immunized once with plasmid DNA as described above either 2, 4, 6 or 8 days later. Control mice were injected with carrier (NaCl) on either day 2 or 6 after tumor challenge. Tumor load of MOCP315.BM.Luc.IgAλ2 was followed longitudinally as described above by measurements of myeloma protein M315 in sera and IVIS imaging.

ELISA for M315 and FLC

Measurement of complete M315 myeloma protein by sandwich ELISA. Coat: anti-Id^{M315} mAb (2 µg/mL, Ab2.1–4) specific for a paratope-related Id that depends on myeloma protein H-L chain assembly for its binding.[30] Serum samples were added, threefold diluted starting at 1:50. Purified M315 served as standard. Detection mAb was anti-mouse IgA (1 µg/mL, BD Pharmingen, San Diego, California, USA).

Measurement of λ2/3 by sandwich ELISA. Coat: 9A8 mAb specific for Vλ1/2.[31] Serum samples were added, threefold diluted starting at 1:50. Purified M315 served as standard. Detection mAb was biotinylated 2B6 specific for Cλ2/3.[31] This ELISA detects free λ2/3 chains as well as λ2/3 chains assembled with H chains. In the current context, since the M315 myeloma protein is IgAλ2, it is assumed that a signal in this ELISA is caused by λ2.

Capture was performed with streptavidin-alkaline phosphatase (GE Healthcare Chicago, Illinois, USA) and developing with phosphatase substrate (Sigma). Optical Density (OD) at 405 nm was measured with a TECAN Sunrise Microplate using the Magellan V.5.03 program.

Limiting dilutions

Single cells were seeded out into single wells in 96 well plates. Wells with single colonies were selected, and cells expanded for 4 days before supernatants were harvested and analyzed by ELISA for secretion of M315 and free λ2 L chains as described above.

Bone marrows were isolated from the femurs of BALB/c mice after immunization (n=1–2). The bone marrows were flushed with fresh medium, and the cells cultured for approximately 1 week, before tumor cells were cloned by limiting dilution as described above.

Epitope predictions

Predictions of epitopes were performed using NetMHCpan 4.0 (DTU Health Tech, Denmark). The entire amino acid sequence of V_H of M315 was used as query, and peptide lengths were set to 8, 9 or 10-mers. Selected alleles were Mouse H2-K^d, D^d and L^d. The standard setting threshold for strong binders (0.5% rank) and weak binders (2% rank) was used.

Statistical analysis

All statistical analyses were performed with GraphPad Prism (V.8). A two-way analysis of variance was used to test significance between groups. Tukey's or Bonferroni multiple comparison tests were used depending on the number of groups compared. Differences in overall survival were tested with Mantel Cox log-rank test. Results were considered statistically significant when $p<0.05$.

RESULTS

DNA vaccines encoding vaccine proteins that target DCs

The general vaccine strategy used here has previously been described.[26] Briefly, plasmids encode polypeptide chains that homodimerizes via a centrally placed dimerization motif, thus forming X-shaped vaccine molecules. At the amino terminal end, the dimeric vaccine proteins contain two identical targeting units specific for APC. At the carboxy terminal end, they contain two identical antigenic units. The APC-targeting and antigenic units can be exchanged by use of the cassette plasmid (figure 1A). On i.m. injection of plasmids, followed by EP of the injection site, the transfected cells secrete vaccine protein dimers that via their targeting units bind APC for improved presentation of antigen to T cells.

The antigenic unit used here is a scFv composed of the V regions of myeloma protein M315 (IgAλ2) produced by the BALB/c mouse MM cell line MOPC315[16] ($scFv^{315}$, figure 1A, left). As an antigen control, we used an irrelevant scFv derived from the BALB/c B lymphoma cell line A20 ($scFv^{A20}$, figure 1A middle). As a targeting unit we used mouse chemokine CCL3 (Mip-1α), previously shown to target chemokine receptors CCR1, 3 and 5 on APC for induction of strong $CD8^+$ and Th1 T-cell responses.[21 32] As a non-targeting control, we used $scFv^{\alpha NIP}$ derived from the B1-8 mAb specific for the hapten NIP (figure 1A right). Plasmid constructs were transiently transfected into HEK293E cells that secreted vaccine proteins with anticipated structure as confirmed by ELISA (online supplemental figure 1).

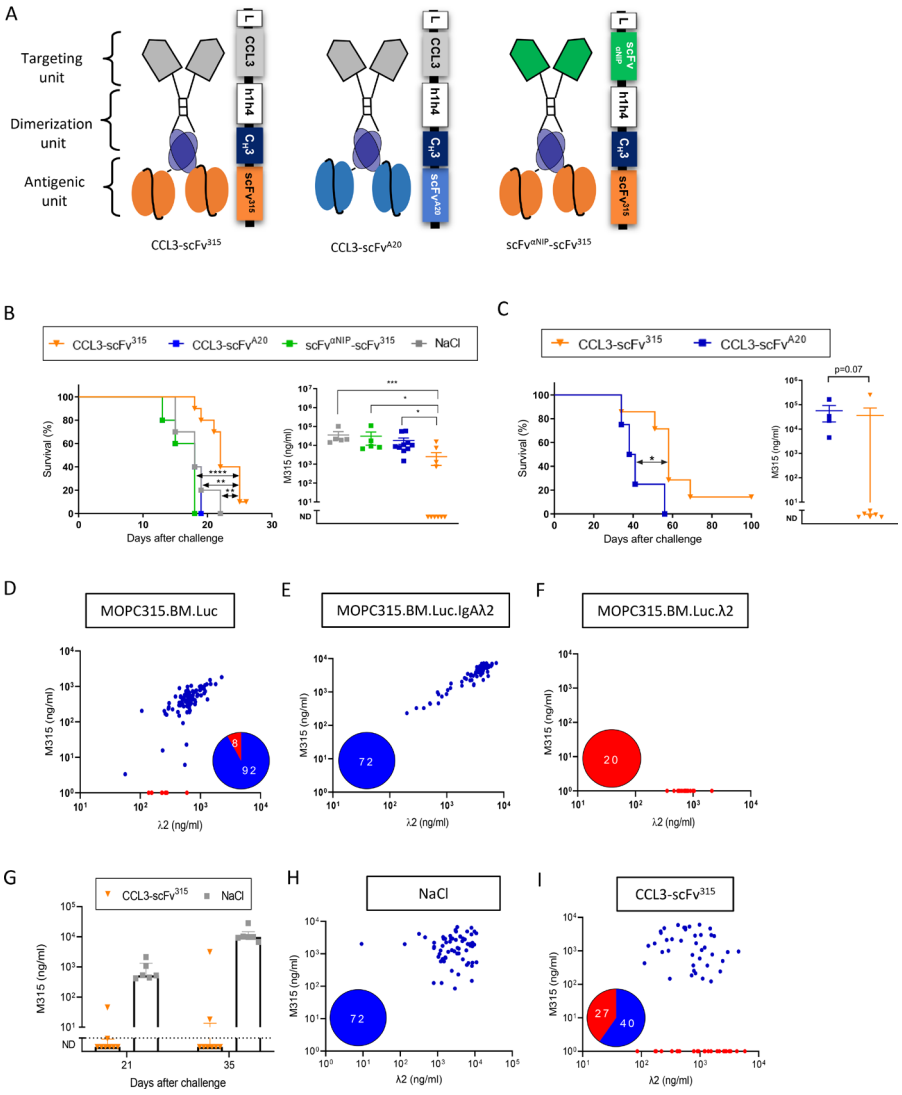


Figure 1 Ig heavy chain-loss variants of MM cells escape tumor-protective immunity induced by Id vaccination. (A) Schematic structure of targeted homodimeric vaccine proteins. The two identical polypeptide chains are held together by a centrally located dimerization unit composed of a shortened hinge (h1h4) and the C_H3 domain from hγ3 Ig H chains. The two targeting units used here are either CCL3 (Mip-1α) or scFv from an anti-NIP mAb ($scFv^{\alpha NIP}$, negative control). The two antigenic units were either $scFv^{315}$ (from the BALB/c MOPC315.BM cell line) or $scFv^{A20}$ (from the BALB/c A20 B lymphoma cell line). The plasmid cassette vector encoding the vaccine protein are indicated to the right. (B) BALB/c mice were immunized as indicated (box), by intramuscular (i.m.) injection of 50 µg of plasmid DNA, immediately followed by electroporation (EP). Fourteen days later, MOPC315.4 tumor cells (1×10^5) were injected s.c. (n=10 for CCL3-$scFv^{315}$ and CCL3-$scFv^{A20}$; n=5 for NIP-$scFv^{315}$ and NaCl). Mice were euthanized when tumor size reached 15 mm. Shown is survival (left) and levels of M315 myeloma protein in sera of vaccinated mice on day 13 after challenge (right, mean±SEM). (C) Mice were immunized i.m./EP with 50 µg of the indicated plasmids. Fourteen days later, MOPC315.BM.Luc (2×10^5 cells/mice) were injected intravenous (n=8 for CCL3-$scFv^{315}$ and n=4 for CCL3-$scFv^{A20}$). End point was paraplegia due to spinal involvement of MM. Showing survival (left) and M315 in sera on day 35 after challenge (right, mean±SEM). (D) Analysis of complete M315 (IgAλ2) or λ2 L chains by ELISA in supernatants of single clones derived from the MOPC315.BM.Luc cell line. Pie chart with embedded numbers indicate number of clones producing complete IgAλ2 (blue) or only λ2 (red). (E) A clone producing complete M315 (MOPC315.BM.Luc.IgAλ2) was cultured in vitro for 14 days, recloned and analyzed for production of M315 and λ2 by single clones. (F) Isolation of FLC-producing MOPC315.BM.Luc.λ2. Tumor cells were cloned from femur BM of a CCL3-$scFv^{315}$ vaccinated and MOPC315.BM.Luc-challenged mouse that developed MM and paraplegia in the absence of serum M315. Single clones were analyzed for production of M315 and λ2. (G–I) Mice were immunized i.m./EP with 50 µg of CCL3-$scFv^{315}$ DNA, or NaCl as control. 2×10^5 MOPC315.BM.Luc.IgAλ2 cells were injected intravenous 14 days after vaccination. (G) M315 levels in sera of mice on day 21 and 35 after challenge (mean±SEM). (H, I) M315 and λ2 in supernatants of single clones obtained from BM of mice 35 days after challenge (n=1). Mice had been immunized with either NaCl (H) or CCL3-$scFv^{315}$ (I). Mean±SEM are shown. Statistics: Kruskal-Wallis with Dunns multiple correction was used when comparing several groups, two-way ANOVA with Bonferroni correction was used for comparing two groups and Mantel Cox Log-Rank test was used for the survival data. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ANOVA, analysis of variance; BM, bone marrow; MM, multiple myeloma; MOPC, mineral oil-induced plasmacytoma; ND, not detected; scFv, single-chain fragment variable.

CCL3-scFv^{315}-immunized mice that succumb to tumor challenge develop FLC MM

BALB/c mice were DNA vaccinated once and challenged with two variants of MOPC315 MM. Surprisingly, both in the s.c. MOPC315.4 model[27] (figure 1B) and in the intravenous MOPC315.BM model[17] (figure 1C), most of the CCL3-scFv^{315}-immunized mice that developed s.c. tumors (figure 1B) or paraplegia due to BM involvement (figure 1C), had undetectable levels of myeloma protein M315 in sera. This discrepancy was not observed in control mice immunized with CCL3-scFv^{A20}. The phenomenon was confirmed in experiments using luciferase-labeled MOPC315.BM.Luc cells injected intravenous and bioluminescence as a read-out for tumor load (online supplemental figure 2). Thus, while radiance and M315 levels correlated strongly in the CCL3-scFv^{A20}-immunized mice, there was no such correlation in the CCL3-scFv^{315}-immunized mice. Of note, immunization with CCL3-scFv^{315} induced anti-M315 antibodies, indicating efficient DNA vaccination (online supplemental figure 3).

Importantly, in the above experiments, we measured M315 by use of an anti-Id^{315} mAb (Ab2-1.4) that needs assembled (H+L chains) myeloma protein for its binding.[30] Thus, an explanation for the discrepancy between tumor growth and serum myeloma protein could be that MM cells in scFv^{315}-immunized mice had lost H chain production, resulting in FLC MM. This would suggest that a way for escaping a CCL3-scFv^{315}-induced immune response could be to abrogate production of myeloma protein H chains.

The MOPC315.BM cells contain a fraction of cells that only secrete FLC

The selective outgrowth of H chain-loss MM cells in CCL3-scFv^{315}-immunized mice challenged with MOPC315.BM could be explained by pre-existing H chain-deficient cells in the tumor cell inoculum. To examine this, we cloned the MOPC315.BM.Luc stock cell used in figure 1C by limiting dilution. Overall, 92% of clones secreted IgAλ2 myeloma protein (figure 1D). We recloned the cells secreting complete IgAλ2 and found that this phenotype was stable for up to 2 months of in vitro culture without emergence of H chain-loss variants (figure 1E and online supplemental figure 4A). Cloned cells that stably secreted complete M315 are henceforth denoted MOPC315.BM.Luc.IgAλ2. The residual 8% of clones secreted λ2 L chains, but not complete M315. Cloned cells that only secreted free L chains and are henceforth denoted MOPC315.BM.Luc.λ2. These FLC MM cells had a stable phenotype on in vitro culture for 2 weeks and recloning (figure 1F). The growth rates in vitro of MOPC315.BM.Luc.IgAλ2 and MOPC315.BM.Luc.λ2 cell lines were comparable with a doubling time of 16–17 hours (online supplemental figure 4B). The two cell lines also grew at comparable speeds in vivo (online supplemental figure 4C,D). Because lack of complete M315 in supernatants of MOPC315.BM.Luc.λ2 cells could be due to either impaired production or secretion, we prepared cell lysates that were analyzed by M315-specific and λ2-specific Western blots and ELISAs. The results show that MOPC315.BM.Luc.λ2 cells do not produce H chains while MOPC315.BM.Luc.IgAλ2 cells do (online supplemental figure 4E,F). Consistent results were obtained by analysis by flow cytometry, since MOPC315.BM.Luc.λ2 cells failed to express M315 on their cell surface while MOPC315.BM.Luc.IgA.λ2 cells did (online supplemental figure 4G). RT-PCR analysis revealed that mRNA expression of V_H^{315} was strongly reduced, although detectable, in MOPC315.BM.Luc.λ2 cells (online supplemental figure 5). On cloning and sequencing, the faint V_H^{315} band had identical nucleotide sequence to that previously reported for V_H^{315}.[19] The mechanism for the strongly reduced H chain mRNA in H chain-loss MOPC315.BM.Luc.λ2 cells remains to be elucidated.

Next, we investigated if the H chain-loss phenomenon could be the result of V_H-specific immunity induced by immunization. Mice were immunized with CCL3-scFv^{315} or NaCl, and 2 weeks later challenged with cloned MOPC315.BM.Luc.IgAλ2 cells. While serum M315 levels increased with time in the NaCl group, levels remained low in CCL3-scFv^{315} immunized mice (figure 1G). On day 35 after challenge, MM cells were flushed from (femur) BM and cloned by limiting dilution. All clones in the NaCl group produced M315, suggesting that H chain loss in MOPC315 cells is infrequent in non-immunized mice in vivo (figure 1H). In striking contrast, 27 out of 67 (40%) of clones from CCL3-scFv^{315}-immunized mice did not produce M315 even though they all produced λ2 L chains (figure 1I). This suggests that H chain-loss variants escape a vaccine-induced immune attack.

Immunized mice are protected against MOPC315.BM.Luc.IgAλ2 while they succumb to the FLC variant

Having developed cloned MOPC315.BM cell lines that either secrete H+L myeloma protein (MOPC315.BM.Luc.IgAλ2) or only free λ2 L chains (MOPC315.BM.Luc.λ2), we tested these two cell lines in challenge experiments. Mice were immunized with CCL3-scFv^{315} and challenged 14 days later with either of the two variants. NaCl was used as a negative control. Six out of eight immunized mice were protected against cells that produced complete M315 (MOPC315.BM.Luc.IgAλ2), as measured by bioluminescence imaging (BLI) (figure 2A,B), serum M315 (figure 2C) and survival (figure 2D). However, two mice had a bioluminescence signal and M315 in their sera (figure 2C); these two mice developed paralysis and did not survive (figure 2D).

In contrast, CLL3-scFv^{315} immunized mice were not protected against cells that only produced free λ2 L chains (MOPC315.BM.Luc.λ2) measured either by BLI (figure 2E–F) or by survival (figure 2H). As would be expected, none of the mice challenged with the FLC MM cells had detectable serum levels of M315 throughout the experiment (figure 2G). These results indicate that H chain-expression by MOPC315 MM cells is required for CCL3-scFv^{315} immunization to have a protective effect. In

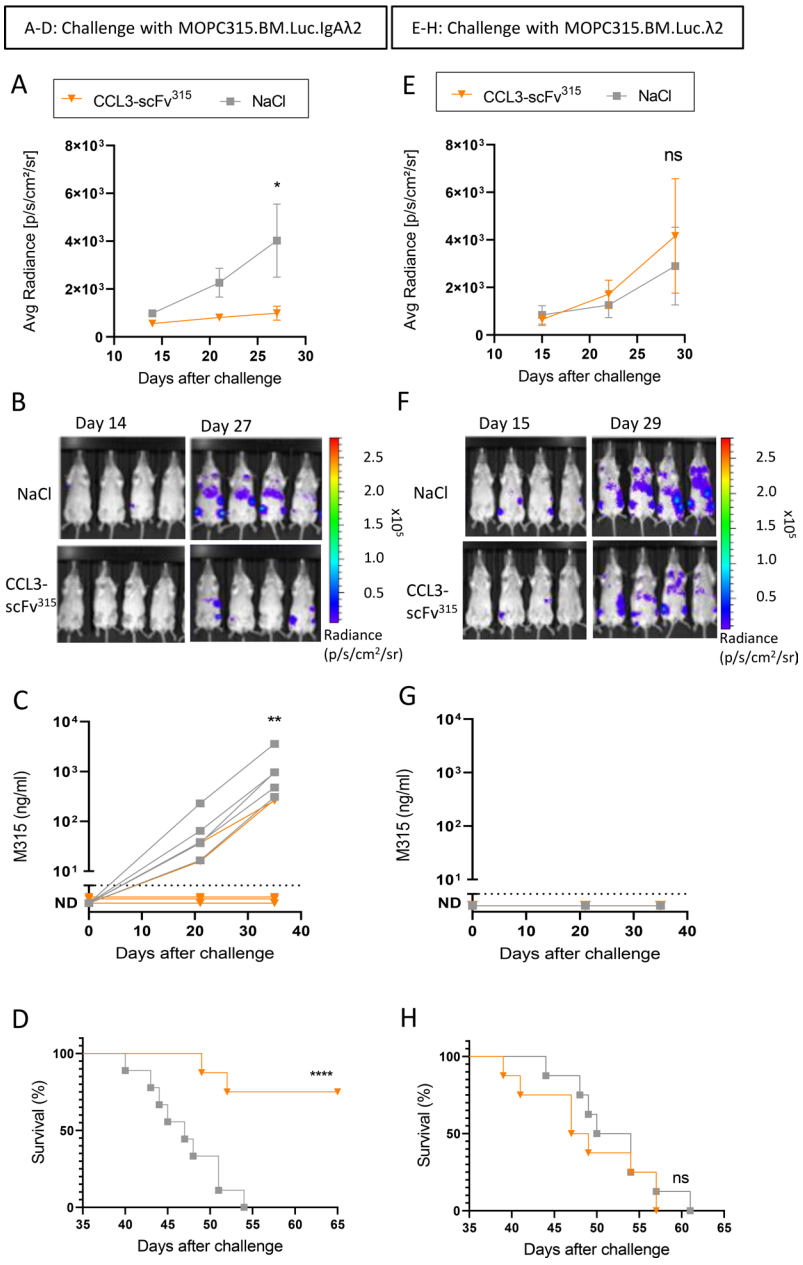


Figure 2 Id-immunized mice are protected against MM cells that produce complete myeloma protein while they succumb to FLC MM cells. (A–H) BALB/c mice were immunized i.m./EP with 50 µg CCL3-scFv^{315} or NaCl and challenged 14 days later with MOPC315.BM.Luc.IgAλ2 cells (2×10^5) that produce complete M315, (left panel), (A–D) or MOPC315.BM.λ2 cells (2×10^5) that produce only free λ2 L chains (right panel), (E–H). (A, E) Images of whole body bioluminescence (BLI) showing mean±SEM, (B, F) representative ventral images, (C, G) serum M315 on days 0, 21 and 35 in individual mice, and (D, H) survival. End point was paraplegia. n=5–8 mice/group. Statistics: Two-way ANOVA with Bonferroni correction (A, C, E, G) and Mantel Cox Log-Rank test for survival (D, H). *p<0.05, **p<0.01, ****p<0.0001. ANOVA, analysis of variance; MM, multiple myeloma; ND, not detected; ns, not significant.

line with this, splenocytes from extensively immunized mice killed MOPC315.BM.Luc.IgAλ2 cells more efficiently than MOPC315.BM.Luc.λ2 cells, in vitro (online supplemental figure 6).

When all performed experiments (n=5) were pooled, out of 23 CCL3-scFv^{315}-vaccinated mice challenged with MOPC315.BM.Luc.IgAλ2, 78% were protected against MM, 9% developed M315 secreting (M315+) MM, while 13% developed H chain-loss MM (ie, FLC MM). In contrast, all (100%) of NaCl-immunized and challenged mice (n=32) developed M315+MM (online supplemental figure 7).

CD8$^+$ T cells are a prerequisite for protection and escape of H chain-loss cells in immunized mice

We next investigated protection and H chain loss in the absence of functional CD8$^+$ T cells. To study this, we used Batf3$^{-/-}$ mice on a BALB/c background. These mice lack cDC1 cells in the spleen,[33] and therefore, fail to develop normal CD8$^+$ T cell responses. Batf3$^{-/-}$ mice were immunized

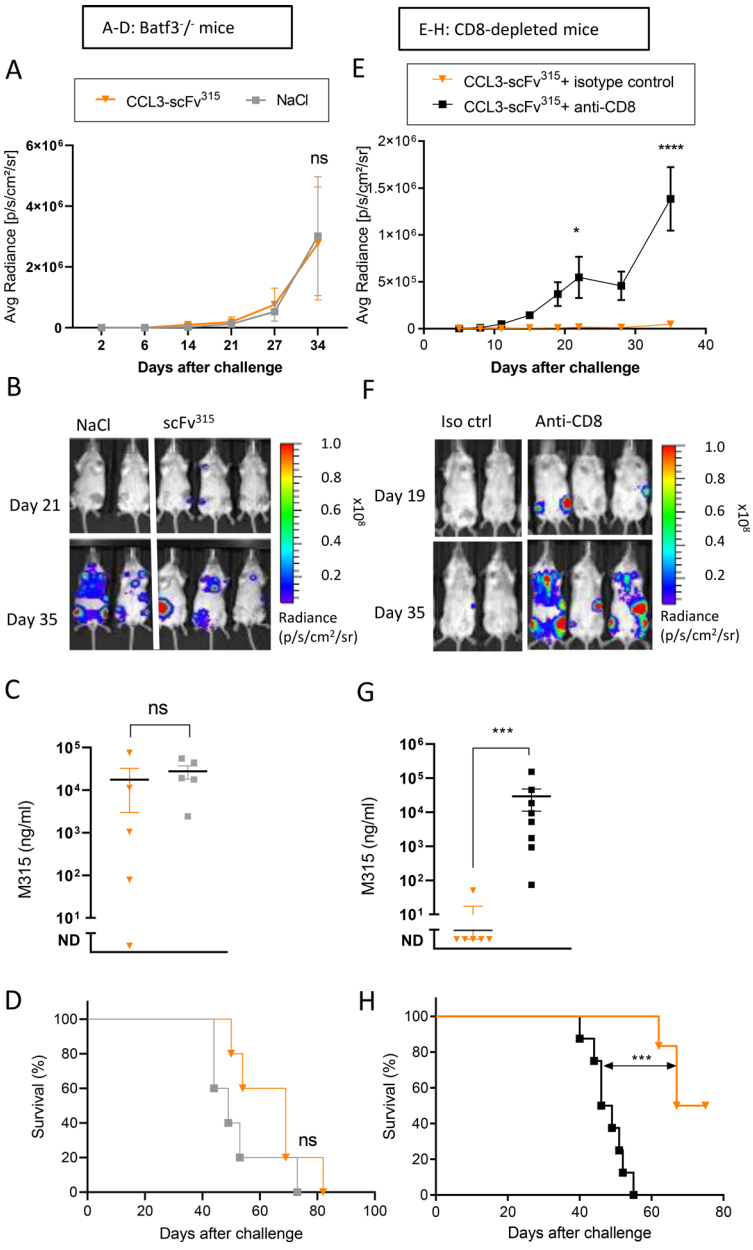


Figure 3 CD8⁺ T cells are required for vaccine-induced protection and H chain loss. (A–D) Batf3$^{-/-}$ BALB/c mice were immunized i.m./EP with 50 µg CCL3-scFv^{315} DNA or NaCL and challenged intravenous 14 days later with 2×10^5 MOPC315.BM.Luc cells. (A, B) Tumor growth was followed by whole body bioluminescence. Representative ventral images are shown. (C) M315 in sera on day 35 (mean±SEM), and (D) survival. (E–H) BALB/c were immunized with CCL3-scFv^{315} and challenged with MOPC315.BM.Luc 14 days later. From day 12 to day 21, mice were injected every other day with 100 µg of either depleting mAb against CD8 or with isotype control mAb. Thereafter, mice were injected with mAbs once a week for 4 weeks. (E, F) Tumor growth was monitored by whole body imaging, (G) measurement of M315 in sera at day 35 (mean±SEM), and (H) survival. A–D: n=5 mice/group, E–H: n=6–8 mice/group. Statistics: Two-way ANOVA with Bonferroni correction (A, E) Mann-Whitney U test (C, G), Mantel Cox log-rank test (D, H). *p<0.05, ***p<0.001, ****p<0.0001. ANOVA, analysis of variance; ND, not detected; ns, not significant; scFv, single-chain fragment variable.

with CCL3-scFv^{315} followed 14 days later with intravenous injection of MOPC315.BM.Luc.IgAλ2 cells. Immunized mice were not protected against tumor development as measured by BLI (figure 3A,B), serum M315 levels (figure 3C) or survival (figure 3D).

The results obtained with Batf3$^{-/-}$ mice suggest that CCL3-scFv^{315} immunization of normal BALB/c mice elicits CD8⁺ T cells that kill MOPC315.BM.Luc.IgAλ2 cells. To further test this idea, we treated CCL3-scFv^{315} immunized BALB/c mice with an anti-CD8 mAb that depletes CD8⁺ T

cells. An isotype-matched mAb was used as a control. On depletion of $CD8^+$ T cells, protection of immunized mice was lost as measured by BLI (figure 3E,F), serum M315 levels (figure 3G) and survival (figure 3H). The fact that all anti-CD8 treated and challenged mice had M315 in their sera showed that anti-CD8 treatment abrogated the H chain-loss phenomenon. These results demonstrate that $CD8^+$ T cells elicited by CCL3-$scFv^{315}$ immunization protect against tumor development and are responsible for the H chain loss and emergence of FLC MM.

Two CD8 epitopes in V_H^{315} that both depend on somatic mutations

V_H^{315} expresses 8 amino acid replacements compared with the closest germline equivalent.[19] We tested if any of these eight expressed mutations could be important for eliciting vaccine-induced $CD8^+$ T cells with protective potential. First, we used the NetMHCpan4.0 algorithm to find peptides that (1) contained an expressed mutation and (2) bound an MHC class I molecule (K^d, D^d, L^d) in the $H\text{-}2^d$ haplotype of BALB/c (figure 4A and online supplemental table 1). Next, we constructed a series of CCL3-scFv vaccine molecules based on $V_H^{germ}V_L^{315}$ where all eight mutations were introduced systematically, one by one, in different vaccine molecules (figure 4B). For reasons of simplicity, $V_H^{germ\ Mut1}V_L^{315}$ is denoted Mut1 and so on. Mice immunized with either Mut1 and Mut5 were completely protected against a challenge with MOPC315.BM.Luc or MOPC315.BM.Luc.IgAλ2. In fact, protection was equal to or even better than that induced by $scFv^{315}$ (figure 4C–E, online supplemental figure 8). The rest of the various Muts induced either no protection or inferior protection compared with that seen with $scFv^{315}$. It should be noted that some of the Muts (2 and 7) were unlikely to stimulate $CD8^+$ T cell responses since the corresponding predicted peptides (figure 4A) were restricted to L^d and since MOPC315.BM.Luc cells only very weakly express L^d compared with K^d and D^d (online supplemental figure 9). Although Mut6 by itself induced some protection, Mut5,6 (where both Mut5 and Mut6 had been introduced in V_H^{germ}) was more protective (online supplemental figure 10). We conclude that amino acid residue 68 can either be isoleucine (I, as in germline) or valine (V, as in V_H^{315}). Physicochemical properties of I and V are similar, and peptides with either residue are predicted to bind well to K^d (0.1589 vs 0.3417, analyzed by NetMHCpan 4.0). Since Mut5,6 is naturally present in the V_H^{315} sequence, we chose to use this immunogen in the experiments to follow.

Since Mut1 and Mut5,6 induced protective immune responses, we asked whether the corresponding peptides were the only ones responsible for full protection. To test this, we produced CCL3 vaccines based on $scFv^{315}$ but where either Mut1 or Mut5,6 were removed either singly, or both of them simultaneously. These constructs are denoted ΔMut1, ΔMut5,6 and ΔMut1,5,6, respectively (figure 5A). As would be expected, since each of Mut1 and Mut5,6 induced protection, either ΔMut1 or ΔMut5,6 alone did not abrogate protection (figure 5B). However, ΔMut1,5,6 failed to induce any protection indicating that Mut1 and Mut5,6 are the major protective CD8 epitopes in the V_H^{315} sequence (figure 5C). Mut1 is predicted to be presented on K^d nanomer (residues 26–34) and D^d nanomer (residues 27–35) while Mut5,6 is predicted to be presented as a decamer on K^d (figure 4A).

It should be noted that most Muts (figure 4C,D and online supplemental figure 8) as well as ΔMut1,5,6 (figure 5) induced some delay of tumor growth compared with the NaCl control. This could be due to a $CD4^+$ T cell response to a well-defined epitope in $V\lambda2^{315}$ immunizing constructs[8 20] and/or weak $CD8^+$ T cell responses to non-Mut1 or non-Mut5,6 CD8 epitopes.

Therapeutic effect of CCL3-$scFv^{315}$ DNA vaccines

To evaluate if vaccination could be therapeutic, we injected BALB/c mice intravenous with MOPC315.BM.Luc.IgAλ2 cells followed by single immunization with CCL-$scFv^{315}$ either 2, 4, 6, or 8 days later. Control mice were immunized with NaCl on either day 2 or 6 (figure 6A). As would be expected, the control mice developed progressive disease as measured by BLI. By contrast, mice immunized on either day 2 or 4 had no evidence of disease up to day 35. Mice immunized on day 6 had reduced but detectable BLI signals while mice immunized on day 8 only had a slight reduction compared with control mice (figure 6A). However, all controls and immunized mice succumbed to MM although disease development was delayed in mice immunized on either day 2 or 4 (figure 6B). On reaching the end point, all NaCl-injected mice had high levels of serum M315. By contrast, half of the mice immunized on either day 2 or day 4 had no M315 in sera despite having developed paraplegia. Thus, 50% of day 2-vaccinated and day 4-vaccinated mice developed FLC MM due to H chain loss (figure 6C,D). This was also the case with one out of four mice that had been immunized on day 6. Thus, CCL3-$scFv^{315}$ immunization had a therapeutic effect but the duration was limited in part due to outgrowth H chain-deficient MM cells.

DISCUSSION

Malignant plasma cells are thought to be derived from B cells that have undergone a GC reaction during which somatic hypermutation occurs.[4–6] Therefore, MM cells should potentially express neoantigenic Id epitopes in their V regions. We here show that Id vaccine-induced $CD8^+$ T cells specific for myeloma protein V_H neoantigens protect and even have a therapeutic effect against MM in the MOPC315.BM mouse model. Moreover, MM cells could escape rejection due to V_H-specific $CD8^+$ T cells by losing their H chain expression, resulting in development of FLC MM.

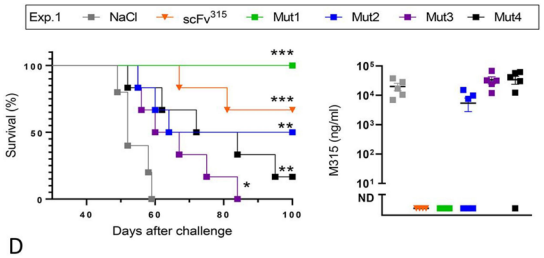
A

Neoepitope	Residue	Mutation	Restriction	Bind level
GYSITSGYF	26-34	1	$H2K^d$	0.4287
YSITSGYFW	27-35	1	$H2D^d$	0.4571
FPGNKLEWL	41-49	2	$H2L^d$	0.3388
GYNPSLKNRV	59-68	5+6	$H2K^d$	0.3417
SENQFFLKL	75-83	7	$H2L^d$	0.4347
YYCAGDNDHL	94-103	8	$H2K^d$	0.3877
AGDNDHLYYF	97-106	8	$H2D^d$	0.4389

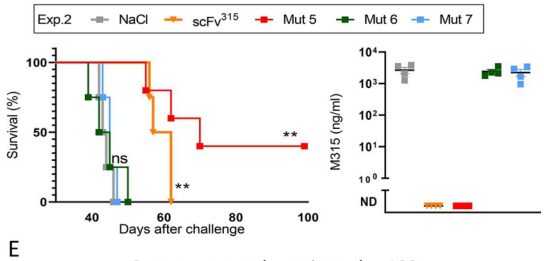
B

V_H in scFv	Amino acid residue 34	49	51	53	59	68	76	98
V_H^{Germ}	Y	M	Y	S	N	I	K	R
V_H^{315}	F	L	F	K	G	V	E	G
$V_H^{Germ\ Mut1}$	F	M	Y	S	N	I	K	R
$V_H^{Germ\ Mut2}$	Y	L	Y	S	N	I	K	R
$V_H^{Germ\ Mut3}$	Y	M	F	S	N	I	K	R
$V_H^{Germ\ Mut4}$	Y	M	Y	K	N	I	K	R
$V_H^{Germ\ Mut5}$	Y	M	Y	S	G	I	K	R
$V_H^{Germ\ Mut6}$	Y	M	Y	S	N	V	K	R
$V_H^{Germ\ Mut7}$	Y	M	Y	S	N	I	E	R
$V_H^{Germ\ Mut8}$	Y	M	Y	S	N	I	K	G

C



D



E

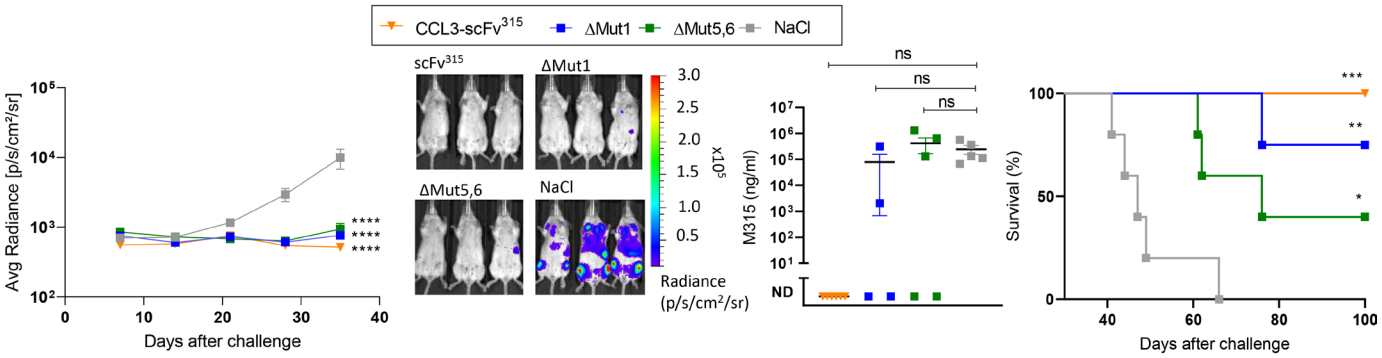
		Status upon euthanasia or day 100 Survival (≥CCL3-scFv315)	Serum M315+ (>1ug/ml)	Heavy chain loss/ mice w/tumor
Exp.1	$scFv^{315}$		0/4	2/2
	Mut 1	+	0/6	0/0
	Mut 2	-	3/6	0/3
	Mut 3	-	5/5	0/5
	Mut 4	-	5/6	0/5
Exp.2	$scFv^{315}$		0/4	4/4
	Mut 5	+	0/5	3/3
	Mut 6	-	4/4	0/4
	Mut 7	-	4/4	0/4
Exp.3	$scFv^{315}$		0/4	1/1
	Mut 8	-	3/4	0/3

Figure 4 Somatic mutations in V_H^{315} are essential for the ability of DC-targeted DNA vaccines to induce protection against MM cells. (A) Overview of neoepitopes predicted using NetMHCpan4.0. Strong binders containing a V_H^{315} mutation are shown. Mutations present in V_H^{315} are highlighted in orange. B) Overview of mutant V_H used in scFv constructs used for immunization. Germline V_H residues are shown in black, V_H^{315}-specific residues in orange. All scFv constructs included V_L^{315}. Below, the constructs are abbreviated as Mut1 etc. (C, D) BALB/c mice were immunized i.m./EP with 50 µg DNA or NaCl i.m. and challenged 14 days later intravenous with (2×10^5) MOPC315.BM.Luc.IgAλ2 (C) or MOPC315.BM.Luc (D), n=4–6 mice/ group. Shown are survival curves (left) and M315 levels in sera at end point (right). (E) Summary of the effects of the different V_H mutations in Exp 1 (figure 4C), Exp 2 (figure 4D) and Exp 3 (online supplemental figure 8) at end point (either day at paraplegia or day 100). Statistics: Mantel Cox log-rank test compared with NaCl group. *p<0.05, **p<0.01, ***p<0.001. MM, multiple myeloma; ND, not detected; ns, not significant.

A

V_H in scFv	Amino acid residue							
	34	49	51	53	59	68	76	98
V_H^{Germ}	... Y ...	M ...	Y ...	S ...	N ...	I ...	K ...	R ...
V_H^{315}	... F ...	L ...	F ...	K ...	G ...	V ...	E ...	G ...
$V_H^{315\Delta Mut1}$	... Y ...	L ...	F ...	K ...	G ...	V ...	E ...	G ...
$V_H^{315\Delta Mut5,6}$	... F ...	L ...	F ...	K ...	N ...	I ...	E ...	G ...
$V_H^{315\Delta Mut1,5,6}$	... Y ...	L ...	F ...	K ...	N ...	I ...	E ...	G ...

B



C

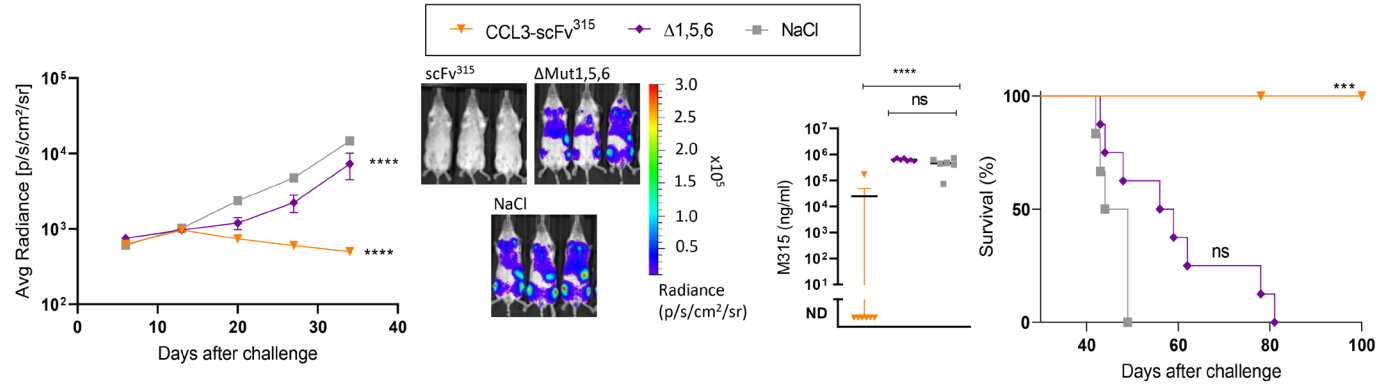


Figure 5 Mut1 and Mut5,6 are the only protective epitopes in the V_H of M315. (A) Overview of mutant V_H used in scFv constructs used for immunization. All scFv constructs included V_L^{315}. Germline is shown in black, M315 in orange. Below, constructs are abbreviated ΔMut1 etc. (B, C) BALB/c mice were immunized with 50 µg DNA or NaCl i.m./EP, and challenged 14 days later intravenous with MOPC315.BM.Luc.IgAλ2 (2×10^5 cells). Shown are bioluminescence measurements (mean±SEM) and representative ventral images on day 35 (left), M315 levels in sera at endpoint (mean±SEM, middle) and survival (right). n=5–8 mice/group. Statistics: Two-way ANOVA with Tukey's post-test (bioluminescence), One-way ANOVA with Tukey's post-test (M315 measurements) and Mantel Cox log-rank test (survival curves) compared with NaCl group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ANOVA, analysis of variance; ND, not detected; ns, not significant; scFv, single-chain fragment variable.

Vaccination was performed with plasmid DNA encoding dimeric CCL3-scFv^{315} fusion protein that targets one or more of CCR1, 3 and 5 on DC.[21 32] Such Id-immunization induced potent V_H^{315}-specific CD8$^+$ T cells, and anti-Id antibodies. However, since removal of CD8$^+$ T cells in vaccinated mice abrogated tumor protection, Id-specific antibodies did not protect against MOPC315.BM on their own.

The vaccine-induced CD8$^+$ T cells were specific for three out of eight mutations expressed by V_H^{315}. It has previously been described that CD4$^+$ T cells are tolerant to germ line-encoded V region sequences but can respond to neoantigenic sequences that express mutations.[7 8 34 35] Neoantigen dependency has been less extensively studied for CD8$^+$ T cells. The present results suggest that also CD8$^+$ T cells are tolerant to germline encoded V region sequences but can respond to V sequences that express mutations.

By doing a series of mutations in the scFv^{315} immunogen, we could demonstrate that CD8$^+$ T cell responses were specific for the $Y^{34}\rightarrow F^{34}$ and the $N^{59}\rightarrow G^{59}/I^{68}\rightarrow V^{68}$ mutations. The residue 34 mutation was predicted to be part of a K^d-restricted peptide GYSITSGYF (residues 26–34) or a D^d-restricted peptide YSITSGYFW (residues 27–35). The 59/68 mutations were predicted to jointly reside on a K^d-restricted decameric peptide GYNPSLKNRV (residues 59–68). It has previously been described that not only nonamers but also decamers can be presented on K^d molecules.[36 37]

The ease by which Id-specific CD8$^+$ T cells were elicited appears to contradict the paucity of Id-peptides eluted

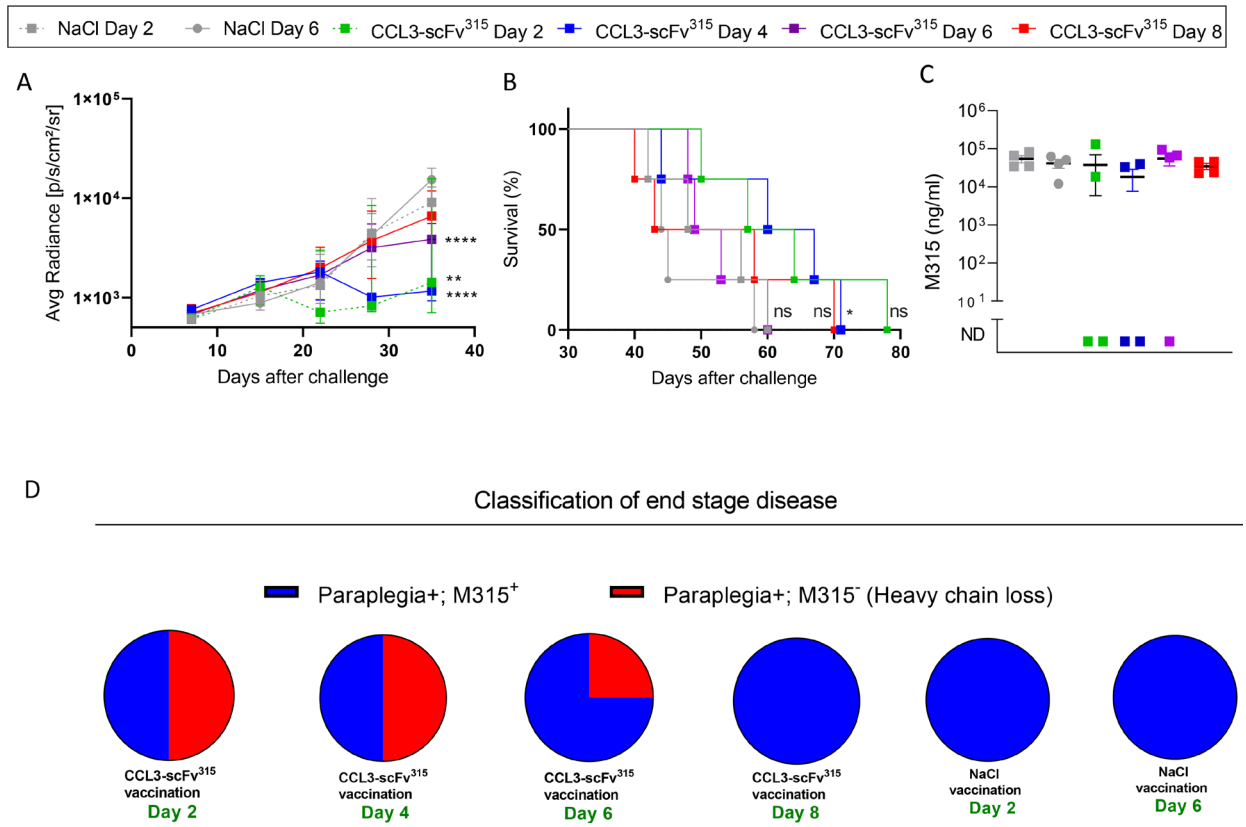


Figure 6 APC-targeted vaccines have a therapeutic effect. (A–D) BALB/c mice were challenged intravenous with MOPC315.BM.Luc.IgAλ2 cells (5×10^4), and immunized i.m./EP with 50 µg DNA or NaCl on the indicated days after challenge. (A) Tumor growth was followed by whole body imaging (mean±SEM), (B) survival and (C) M315 levels in sera at end point (mean±SEM). (D) Pie chart classification of end stage disease. n=4 mice/group. Statistics: Two-way ANOVA with Tukey's post-test (bioluminescence) and Mantel Cox log-rank test (survival curves) compared with NaCl group. *p<0.05, **p<0.01, ****p<0.0001. ANOVA, analysis of variance; ND, not detected; ns, not significant; scFv, single-chain fragment variable.

from MHC class I molecules in human B cell malignancies.[12 13] To resolve this issue, more MM patients should be investigated in both functional and elution studies. Also, MM patients should be more extensively investigated for myeloma protein V region neoepitopes that could fit their MHC molecules. In the meanwhile, the present results are clearly encouraging for Id-vaccination in MM since V regions in post GC MM cells are likely to be a rich source of neoantigenic Id epitopes.

Interestingly, in CCL3-scFv^{315}-immunized mice, H chain-deficient MM cells escaped killing and grew into FLC MM. Whether V_H-reactive CD8$^+$ T cells could contribute to development of FLC MM in humans is an open question. After all, most MM patients that develop FLC MM have not been deliberately Id-immunized. It might be, however, that certain treatments of MM could enhance immunogenicity of myeloma protein V regions, thereby causing induction of V_H-reactive CD8$^+$ T cells.[38 39]

The molecular mechanism by which H chain production is turned off should be an interesting topic for investigation. We show that in H chain-loss MOPC315.BM.Luc.λ2 cells, mRNA encoding the V_H^{315} is strongly reduced, but the mechanism remains to be elucidated. The mechanism for H chain loss and FLC MM in patients may in most cases be contributed by defects at the DNA level.[40]

The emergence of FLC MM in CCL3-scFv^{315}-vaccinated mice challenged with MOPC315.BM is surprizing for several reasons: (1) Id-specific CD4$^+$ T cells are known to recognize a mutated Id-sequence (residues 91–101) on λ2^{315} L chain of M315 myeloma protein,[8 41] and this epitope is present in scFv^{315} antigen used for vaccination. (2) Previous studies have shown that TCR-transgenic mice expressing high amounts of the Id (λ2^{315})-specific CD4$^+$ T cells reject MOPC315 in the absence of B cells or CD8$^+$ T cells.[42 43] Apparently, tumor-infiltrating macrophages endocytose secreted myeloma protein and present pId:MHCII to Id-specific Th1 cells, resulting in IFNγ-dependent induction of M1 macrophages that kill MHCII^{NEG} MOPC315 cells by an NO-dependent mechanism.[20 44 45] (3) FLC-producing variant cells (MOPC315.26) that secrete free λ2^{315} L chains, were killed in Id-specific TCR-transgenic mice.[46] Why, then, did FLC MM emerge in CCL3-scFv^{315}-vaccinated mice? A likely explanation is that the current Id-vaccination does not induce sufficient numbers of Id-specific CD4$^+$ T cells to eradicate the FLC-producing MOPC315 cells. Indeed, Id(λ2^{315})-specific CD4$^+$ T cells are of exceedingly low frequencies[8] and have a severely limited TCR-repertoire,[47] making it difficult to detect these cells in immunized mice. Thus, vaccine-induced Id (λ2^{315})-specific CD4$^+$ T

cells could simply be too few to eliminate FLC MOPC315.BM cells indirectly via macrophages. However, they could be present in sufficient numbers for efficiently helping of V_H^{315}-specific $CD8^+$ T cells via CD4-CD8 T cell collaboration. Supporting this possibility, Id-specific $CD4^+$ T cells elicited by potent immunization with Id($\lambda 2^{315}$)-peptide helped $CD8^+$ T cells specific for undefined non-Id neoantigens, resulting in rejection of MOPC315.BM cells.[48]

Importantly, we show that therapeutic CCL3-scFv^{315} DNA vaccination caused retardation of disease development and emergence of FLC MM. This indicates that the current type of Id-vaccination could be useful for MM patients. In this respect, DNA vaccines similar to the present vaccines, but using human CCL3 (LD78b)[49] and jet delivery, are currently being tested as neoantigen vaccines in humans with a variety of cancers.[50] Efficacy of CCL3-targeted DNA vaccination of MM patients might be improved by multiple immunizations, more powerful means of inducing Id-specific $CD4^+$ T cells and inclusion of check point inhibitors.

In summary, we show that potent V_H neoantigen-specific $CD8^+$ T cells elicited by CCL3-targeted Id-vaccination can have a therapeutic effect in a mouse MM model. Moreover, MM cells can escape rejection by losing expression of the H chain, thus giving rise to FLC MM.

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Competing interests BB is an inventor of patents related to Vaccibody vaccines. He is leader of the Scientific Advisory Board in the Nykode Therapeutics (former Vaccibody AS) company in which he holds shares.

Patient consent for publication Not applicable.

Ethics approval Animal handling and procedures were approved by the Norwegian food safety authority (Mattilsynet, FOTS ID 13893). Imaging and cardiac blood withdrawal were performed under anesthesia.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. All data relevant to the study are included in the article or uploaded as online supplemental information. Data and material not included in the manuscript will be made available to qualified academic researchers on request to the corresponding authors (RB and BB).

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