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Antibody–peptide–MHC fusion conjugates target non-cognate T cells to kill tumour cells

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Abstract

Attempts to generate robust anti-tumour cytotoxic T lymphocyte (CTL) responses using immunotherapy are frequently thwarted by exhaustion and anergy of CTL recruited to tumour. One strategy to overcome this is to retarget a population of virus-specific CTL to kill tumour cells. Here, we describe a proof-of-principle study using a bispecific conjugate designed to retarget ovalbumin (OVA)-specific CTL to kill tumour cells via CD20. A single-chain trimer (SCT) consisting of MHC I H-2K^b/SI-INFEKL peptide/beta 2 microglobulin/BirA was expressed in bacteria, refolded and chemically conjugated to one (1:1; F2) or two (2:1; F3) anti-hCD20 Fab' fragments. In vitro, the [SCT × Fab'] (F2 and F3) redirected SIINFEKL-specific OT-I CTL to kill

CD20⁺ target cells, and in the presence of CD20⁺ target cells to provide crosslinking, they were also able to induce proliferation of OT-I cells. In vivo, activated OT-I CTL could be retargeted to kill [SCT × Fab']-coated B cells from hCD20 transgenic (hCD20 Tg) mice and also EL4 and B16 mouse tumour cells expressing human CD20 (hCD20). Importantly, in a hCD20 Tg mouse model, [SCT × Fab'] administered systemically were able to retarget activated OT-I cells to deplete normal B cells, and their performance matched that of a bispecific antibody (BsAb) comprising anti-CD3 and anti-CD20. [SCT × Fab'] were also active therapeutically in an EL4 tumour model. Furthermore, measurement of serum cytokine levels suggests that [SCT × Fab'] are associated with a lower level of inflammatory cytokine release than the BsAb and so may be advantageous clinically in terms of reduced toxicity.

Keywords

Antibody–peptide–MHC conjugates; Retargeting non-cognate cytotoxic T cells; Single-chain trimer; Immunotherapy

Introduction

Recent results in a range of solid tumours have brought optimism that immunotherapy has a role to play in managing cancer. Historically, the ability of the graft-versus-leukaemia response following allogeneic haematopoietic cell transplantation to eradicate leukaemia has provided strong evidence that T-cell cytotoxicity can be harnessed as a therapy [1]. This belief has been bolstered by the FDA approval of the Sipuleucel-T vaccine which although therapeutically modest is the first cancer vaccine to reach this critical stage [2].

Promising results have also come in the field of adoptive T-cell transfer, where a 50–72 % response rate has been achieved in melanoma patients treated with ex vivo expanded autologous tumour infiltrating lymphocytes (TIL) [3–5]. As an alternative to expanding anti-tumour T cells ex vivo, and to broaden the scope of adoptive transfer, the introduction of genes for tumour antigen-specific TCR has been developed as a way of conferring specificity on a patient's own T cells and thus enabling them to attack tumour cells [6]. Using this approach, responses have been observed in melanoma, metastatic colorectal cancer, and synovial cell carcinoma [7–11], albeit with some severe autoimmune side effects. Finally, the capacity of CTL to destroy bulk tumour has been underlined in a most convincing manner by work of Carl June and colleagues using adoptive transfer of autologous T cells in CLL patients after transduction ex vivo with a CD19-specific chimeric antigen receptor (CAR) [12]. These are recombinant receptors consisting of a scFv fragment recognising a tumour antigen, linked to a hinge spacer, a transmembrane domain, and various intracellular signalling domains to allow triggering of T-cell effector function. The CAR used in this study included a signalling element from the 4-1BB co-receptor, which is known to sustain T cells during immune activation. Once in the patients, the T cells underwent marked expansion and were able to delete tumours of perhaps 10¹² cells and deliver sustained complete responses.

While these clinical data underline the potency of CTL against tumour, tailor-made treatments with ex vivo manipulation of effector cells are likely to prove prohibitively expensive on a large scale. An alternative strategy is the idea of activating and re-directing endogenous T cells. One way to do this is to use bispecific antibodies (BsAb) comprising anti-CD3 and anti-tumour antigen moieties. Unfortunately, this is frequently associated with severe toxicity due to the release of a plethora of inflammatory cytokines [13]. Nevertheless, interest in the field has been maintained with a new class of clinical reagent, single-chain bispecific T-cell engagers (BiTEs), which consist of fused scFv domains from an anti-

tumour mAb and an anti-CD3 mAb, now in development. The first BiTE, blinatumomab, with specificity for CD19 and CD3 has been trialled as a single agent in non-Hodgkin's lymphoma [14] and ALL [15] with objective clinical responses and acceptable toxicity. Trials with BiTE specific for EpCAM, an antigen widely expressed on human adenocarcinoma [16] and cancer stem cells [17], have recently been initiated [18].

A refinement of this strategy is to retarget an existing population of CTL of a single specificity, such as for a particular viral antigen. This has the potential advantage that it avoids the use of anti-CD3 which is non-discriminatory in terms of T-cell recruitment and is likely to trigger cells which are not helpful as effectors but which contribute to the cytokine release syndrome which hamper this approach. To evaluate the potential of this approach, we have expressed the OVA-derived peptide SIINFEKL together with beta-2 microglobulin and the mouse MHC I heavy chain H-2K^b as a single-chain trimer (SCT) [19, 20] and chemically conjugated this to an anti-hCD20 Fab' fragment using the crosslinker, succinimidyl-4-(*N*-male-imidomethyl) cyclohexane-1-carboxylate (SMCC). In this report, we present a proof-of-principle study in which we use this conjugate to re-direct OVA-specific CTL to kill hCD20-expressing cells both in vitro and in vivo.

Materials and methods

Animals and cells

C57BL/6 mice were bred and maintained in local facilities; hCD20 Tg mice have been described previously [21]. Animal experiments were conducted according to the UK Home Office welfare guidelines under Home Office licence PPL30/2451 and approved by the University of Southampton Ethical Committee.

Daudi cells were obtained from the European Collection of Cell Cultures (ECACC) and the B16-F10 melanoma, EL4 and EG7 lines from the American Type Culture Collection (ATCC). EL4 and B16-F10 cells were stably transfected with hCD20 and the resulting lines (EL4h20 and B16h20) maintained in medium with 10 µg/ml puromycin.

Antibodies and reagents

AT80 is an in-house anti-hCD20 mAb [22]; 3/23 (rat anti-mouse CD40) and 25-D1.16 (D1) (anti-H-2K^b-SIINFEKL) [23] were originally gifts from Gerry Klaus (National Institute of Medical Research, London) and Ronald Ger-main (NIH, Bethesda), respectively. Purified IgG was prepared as described previously [24]. Fluorescently conjugated mAb were from BD Biosciences, AbDSerotec, or in-house. SIINFEKL tetramer was from BD Biosciences or in-house. Flow cytometry was performed on a FACSCali-bur and analysed using Cellquest software (BD Biosciences).

Production, refolding, and purification of SCT

KRX *Escherichia coli* cells (Promega, UK) were used for the expression of protein encoded by the plasmid pET21a containing the DNA construct MHCI H-2K^b/SIINFEKL peptide/ beta 2 microglobulin/BirA (SCT) after induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Biolone, UK) and 0.1 % w/v L-rhamnose monohydrate (Promega, UK). Bacterial cells were disrupted by sonication in 20 mM Tris/HCl pH 8.0, 5 mM EDTA, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mg/ml lysozyme, 20 mg/ml DNAaseI with 10 mM MgCl₂ to extract the inclusion bodies, which were then washed before solubilisation in 50 mM Tris/HCl pH 8, 8 M urea, 20 mM 2-mercaptoethanol(2-ME), 50 mM NaCl, 1 mM EDTA. Solubilised inclusion bodies were refolded by dilution using the redox-shuffling refolding buffers described by Lybarger et al. [25] (100 mM Tris, 400 mM L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidised glutathione, 0.1 mM

PMSF pH8). 50 mg of unfolded SCT protein was added in 10 mg aliquots over 24–48 h to 1 l of continuously stirred refolding buffer at 4 °C. After 48–72 h, the mixture was filtered to remove aggregates, concentrated and dialysed against 0.2 M Tris/HCl pH 8.0 buffer at 4 °C. The refolded SCT was purified by size exclusion chromatography using two in series 94.3 × 1.6 cm Superdex 200 (GE Life Sciences, UK) columns attached to a Uvicord SII spectrophotometer and 15 min fractions collected over 24 h. Fractions corresponding to distinct protein peaks were combined, concentrated, and analysed by HPLC.

Tetramer formation

Refolded SCT was biotinylated enzymatically using BirA enzyme (Avidity, USA) separated by size exclusion, analysed by HPLC, and biotinylation confirmed by Western blot. To form tetramers, streptavidin–phycoerythrin (Sigma-Aldrich) was added to biotinylated SCT in a 1:4 molar ratio and tetramer formation confirmed by HPLC and binding to OT-I T cells by flow cytometry.

Production and isolation of [SCT × Fab]

AT80 F(ab)₂ produced from parent IgG by pepsin digestion [26, 27] was reduced with 20 mM 2-ME for 30 min at 30 °C. Fab_{SH} was purified by size exclusion chromatography, collected under nitrogen, and kept on ice. SCT was incubated with SMCC at 20-fold molar excess, for 30 min at 30 °C to obtain maleimide-activated SCT (SCT_{mal}). Excess SMCC was removed by size exclusion chromatography, the SCT_{mal} added to the Fab_{SH}, and the mixture concentrated and left for 6–16 h at 4 °C. 20 mM 2-ME was added to reduce unreduced or re-oxidised homodimers of F(ab')₂ and then excess iodoacetamide to prevent re-oxidation. The SMCC-conjugated products were separated by size exclusion chromatography.

Production of [anti-CD3 × anti-hCD20] bispecific F(ab')₂ (BsAb)

This was produced as described previously [26, 27] using AT80 IgG and the anti-CD3 mAb KT3.

Adoptive transfer of OT-I cells and induction of OVA-specific CTL in vivo

OVA-specific CTL were induced after adoptive transfer of OVA-specific H-2K^b-restricted TCR transgenic T cells from OT-I mice into wild-type C57BL/6 recipients: lymph node and spleen cells were prepared from OT-I mice and 5×10^5 OT-I cells injected i.v. into recipients. 24 h later, mice were immunized by i.p. or i.v. injection of OVA (5 mg) and anti-CD40 mAb (500 µg). After 5 days, total splenocytes were used as effector CTL (typically 20–25 % OT-I cells). For some experiments, mice were immunised with OVA and anti-CD40 in the same way to induce an endogenous T-cell response. Immunised mice were used as a source of splenocyte effectors for the in vitro cytotoxicity assays (described below) or as recipients in the in vivo assays. For the hCD20 Tg B-cell depletion experiments, OT-I cells were transferred into hCD20 Tg recipients, which were then immunised as described above.

In vitro cytotoxicity assays

The assay was modified from previous work [24]. The effectors were splenocytes from OT-I recipients taken at day 5 post-immunisation (above). Targets were labelled with ⁵¹Cr and incubated with retargeting construct and effector cells for 6 h at 37 °C. Supernatant was harvested for the measurement of ⁵¹Cr release. Assays were performed in triplicate, and the maximum release of ⁵¹Cr was determined by adding 150 µl 1 % Nonidet P-40 to target cells. The percentage of specific ⁵¹Cr release was calculated using the formula: % specific release = [(sample release – background release)/(maximum release – background release)] × 100.

In vivo cytotoxicity experiments

For experiments with hCD20 Tg B-cell targets, splenocytes from hCD20 Tg (target) and WT (non-target) mice were labelled with 5 and 0.5 μ M carboxyfluorescein succinimidyl ester (CFSE; Invitrogen), respectively, mixed (1:1), then coated with 5 μ g/ml of retargeting construct at room temperature for 30 min, washed, and injected i.v. ($1-3 \times 10^6$) into OVA and anti-CD40 immunised recipients (described above). Sixteen hours later, splenocytes were analysed by flow cytometry to determine the ratio of target to non-target CFSE-labelled B cells. For the experiments with EL4h20, EL4h20 cells (target) were labelled with 5 μ M CFSE and 20 μ M PKH26 (Sigma-Aldrich) and EL4 cells (non-target) with 5 μ M CFSE only, mixed, coated with 5 μ g/ml retargeting construct and injected (1×10^7) into the peritoneal cavity of immunised recipients. Sixteen hours later, peritoneal cavity cells were harvested to determine the ratio of target: non-target cells.

Lung protection assay

Mice immunized with OVA and anti-CD40, and on day 7, received 3×10^5 B16h20 cells coated with 5 μ g/ml retargeting construct. On day 28, mice were killed and the number of pigmented colonies on the surface of the lungs counted.

In vitro proliferation of CTL

OT-I cells were purified by MACS negative selection (Miltenyi Biotec) and 5×10^4 cells/well were incubated with retargeting construct and irradiated Daudi (1×10^5 or 1×10^4) or EG7 (1×10^5) cells for 72 h. 1 μ Ci [3 H] thymidine per well was added for the final 18 h and cells harvested and incorporation measured. Alternatively, the same conditions were used except OT-I cells were labelled with 2 μ M CFSE prior to incubation. After 72 h, cells were labelled with APC-anti-mouse CD8, and CFSE dilution was determined by flow cytometry.

B-cell depletion

hCD20Tg mice immunized 6 days previously with OVA and anti-CD40 (above) received an i.v. injection of retargeting conjugate. B cells remaining in the blood were assessed by cell count and flow cytometry using APC-anti-CD19 and PerCP-Cy5.5 anti-B220 [21]. The absolute number of B cells/ml blood at each time point was calculated and plotted as a percentage of the starting number.

Serum cytokine assay

Serum from the B-cell depletion assays was taken for the assay of serum cytokines using a Meso Scale Discovery Mouse TH1/TH2 9-plex Assay (Meso Scale Discovery, US) as per manufacturer's instructions. Data were analysed using the supplied MSD Discovery Workbench analysis software.

Statistical analysis

Differences between experimental groups were analysed using a 2-tailed *t* test and survival data using a Log-rank (Mantel Cox test), both performed using GraphPad Prism software.

Results

Production of SCT and its conjugation to anti-hCD20

We have produced a conjugate consisting of anti-hCD20 Fab' chemically linked to a SCT, termed [SCT $\times$ Fab']. Anti-hCD20 Fab' rather than IgG was used to avoid any interaction of the conjugate with Fc receptors. The SCT component, comprising SIINFEKL peptide, beta2 micro-globulin, and H-2K^b domains ($\alpha 1-\alpha 3$), joined by flexible gly/ser linkers to yield a

fusion protein with a BirA biotinylation consensus sequence (Fig. 1a), was expressed in KRX *E. coli*. Figure 1b shows SDS-PAGE analysis at various stages of the production. Following IPTG induction, bacterial lysate (lane 1) was centrifuged to obtain supernatant (lane 2) and insoluble inclusion bodies, which were washed to obtain SCT (lane 3). SCT were refolded by dilution using a redox-shuffling system [25] and further purified by size exclusion chromatography. A sample of the SCT was biotinylated and combined with streptavidin-PE and the binding of the resulting tetramers to OT-I cells assessed (Fig. 1c, upper plots). These bound to OT-I cells at a level comparable with that of a commercial tetramer (Fig. 1c, lower plot).

The SCT and anti-CD20 Fab' molecules were conjugated using the heterobifunctional crosslinker SMCC. Since the SMCC can crosslink the reduced sulfhydryl group within the hinge region of the Fab' heavy chain with any amine group on the SCT, more than one Fab' can be linked to each SCT. Size exclusion chromatography was used to purify [SCT $\times$ Fab'] conjugates with ratios of 1:1 and 2:1 Fab':SCT, termed F2 and F3, respectively. The unconjugated fraction of mixed SCT and Fab' is termed F1. Figure 2a shows SDS-PAGE analysis of the parent F(ab')₂, the unconjugated fraction, and the F2 and F3 conjugates. On reduction, parent F(ab')₂ (lane 2) was dissociated into its heavy (Fd) and light chain components (lane 7). Following conjugation, under non-reducing conditions, the unconjugated components ran as expected for alkylated Fab' and SCT, approximately 50 kD (lane 3); on reduction, the Fd and light chains of the Fab' dissociated, with the SCT unchanged (lane 8). The unreduced F2 conjugate ran at approximately 100 kD as expected (lane 4), with the F3 somewhat larger (lane 5); reduction of F2 and F3 yields light chain, with an associated drop in the molecular weight of the major band, consistent with the Fd region being conjugated to SCT by a non-reducible SMCC linkage (lanes 9 and 10). HPLC analysis showed that the F2 and F3 conjugates ran as a single peak of greater than 95 % purity. Typical yields for the overall process were as follows: 1 l of bacterial induction yielded 100 mg of inclusion bodies. 100 mg of inclusion bodies yields 3–5 % of refolded protein post-purification. A 10 mg SCT $\times$ 10 mg Fab conjugation yielded 2 mg F2 and 1 mg F3 giving a 15 % conjugation efficiency.

To confirm that both arms of the conjugates retained activity, binding to CD20⁺ Daudi cells was assessed; binding via the anti-hCD20 Fab' arm was detected using FITC-25D1 mAb (specific for H-2K^bSIINFEKL) (Fig. 2b). The results indicate that F3 binds with a higher avidity than F2, with half-maximal binding at below 1 μ g/ml (6.66 nM) and greater than 2.5 μ g/ml (25 nM) for F3 and F2, respectively. This most likely reflects the ability of F3 to bind bivalently to Daudi cells compared with the univalent binding of F2. The binding avidity of the F3 conjugate is similar to that previously observed in this laboratory for the parent AT80 F(ab)₂ [22] suggesting that most of the binding activity is retained during the production and processing of the conjugate. When hCD20 on the Daudi cells was blocked with anti-CD20 mAb (AT80), binding of the conjugates was prevented, confirming the specificity of F2 and F3 for hCD20. The dashed line shows the binding of FITC-25D1 to EG7 cells, a H-2K^b mouse T-cell lymphoma line transfected with OVA which presents the H-2K^bSIINFEKL peptide-MHC (pMHC) complex. This indicates that F2 and F3 are able to coat the cells with a higher level of pMHC than is present on the surface of a cell following endogenous processing and presentation of OVA.

Conjugates can redirect OVA-specific CTL to kill tumour cells in vitro

The ability of the [SCT $\times$ Fab'] conjugates to redirect anti-OVA CTL to kill tumour cells was first assessed in vitro. To induce a population of effectors, mice were adoptively transferred with OT-I cells and then immunised with OVA and anti-CD40 mAb 24 h later. After 5 days, total splenocytes were used as effector CTL. The results in Fig. 3a show the

ability of the F2 and F3 conjugates to target these CTL to kill Daudi cells compared with an [anti-CD3 $\times$ anti-hCD20] bispecific F(ab')₂ (BsAb). All three derivatives mediated killing of target cells, with a hierarchy in the order BsAb > F3 > F2. The greater efficacy of the BsAb probably reflects its ability to recruit from a larger pool of effectors as it is able to retarget both OT-I and non-OT-I T cells. The F3 conjugate gave a higher overall level of killing than the F2 conjugate and was also more potent, consistent with its ability to bind to the target bivalently. The absence of killing with irrelevant effectors (Fig. 3a) and in the presence of a mAb blocking either the conjugate:effector (25D1) or conjugate:target (anti-CD20) interaction (Fig. 3b) confirms that this is specific retargeting of SIINFEKL-specific CTL to hCD20 on the Daudi targets [28].

Binding of conjugates induces proliferation of OVA-specific CTL

We next investigated whether the F2 and F3 conjugates could also induce proliferation of the OVA-specific CTL population. As both F2 and F3 have only one arm specific for the T-cell receptor they should not induce T-cell activation in their soluble form. However, when bound to hCD20⁺ target cells, they have the potential to crosslink the TCR and so induce proliferation. As TCR:pMHC interactions are typically of much lower affinity than antigen:antibody interactions, we predict that in this system, the anti-hCD20 arm would bind to target cells first. The array of pMHC presented on the target cell surface would then display a much higher avidity for SIINFEKL-specific CTL. Proliferation was assessed using [³H]thymidine incorporation and CFSE dilution. OT-I T cells were incubated with F2 and F3 in the presence or absence of irradiated Daudi cells. As a positive control, OT-I cells were incubated with EG7 cells. The [³H]thymidine incorporation assay (Fig. 4a) showed that in the presence of Daudi cells, both F2 and F3 and the [anti-CD3 $\times$ anti-hCD20] BsAb are able to induce OT-I proliferation, with greater proliferation in the presence of a higher concentration of Daudi cells. There was no proliferation in the absence of Daudi cells confirming it is dependent on the crosslinking of the TCR via F2 or F3 bound to CD20 on the Daudi cells. With CD8 T cells from control mice, proliferation was only observed with the BsAb, showing the antigen specificity of the response induced by the F2 and F3 (data not shown). Similar results were obtained using CFSE dilution as a measure of proliferation (Fig. 4b). Thus, it is possible that in vivo the constructs have the capacity to induce the expansion of their target CTL in addition to re-directing them to kill tumour cells.

OVA-specific CTL will kill conjugate-coated huCD20⁺ targets in vivo

We next wanted to demonstrate that the conjugates were able to harness an anti-OVA T-cell response to kill target cells in vivo. The CTL response was induced either after adoptive transfer of OT-I cells or from endogenous T cells by immunisation with OVA and anti-CD40.

The first targets were splenocytes from hCD20 Tg mice expressing hCD20 on all of their B cells. hCD20 Tg and control (non-target) splenocytes labelled with 2 and 0.2 μ M CFSE respectively were mixed before the addition of F2 or F3 or [anti-CD3 $\times$ anti-hCD20] BsAb and then injected into immunised mice. After 20 h, the ratio of target to non-target cells in the spleens was determined by flow cytometry. In immunised mice, the F2 and F3 conjugates mediate impressive killing (>90 %) of the hCD20⁺ target cells; this was achieved in recipients after receiving OT-I cells (typically 20–25 % OT-I cells) (Fig. 5a) or with an endogenous response (1–2 % SIINFEKL-specific CD8 cells in the spleen) (Fig. 5b); this matched the performance of the BsAb which was able to mediate killing in both immunised and naïve recipients, reflecting its ability to recruit from the total CD8 population.

We next looked at the in vivo killing of two mouse tumour lines transfected with hCD20: the thymoma EL4h20 (Fig. 5c) and the melanoma B16h20 (Fig. 5d) which express relatively

high and low levels of hCD20, respectively (insets to the panels). EL4h20 targets were labelled with CFSE and PKH26, and EL4 non-targets with CFSE alone, coated with conjugate and injected into the peritoneal cavity of immunised OT-I recipients. Twenty hours later, the ratio of target to non-target cells in the peritoneal cavity was determined (Fig. 5c). The F2 and F3 conjugates performed similarly in this model, with a dose-dependent killing of the target cells. The killing was comparable with that obtained with EG7 cells (express surface pMHC) as targets. For the B16h20 model, we looked at the targeting of an endogenous CTL response; B16h20 cells coated with conjugate were injected i.v. into immunised recipients, and 28 days later, the number of pigmented colonies on the surface of the lungs counted (Fig. 5d). Significantly, fewer colonies formed on the lungs with both F2 and F3 conjugates, with almost complete inhibition of colony formation which matched that obtained in naïve mice with BsAb-coated cells, thus showing that the conjugates can mediate killing of cells expressing a low level of target antigen.

Systemically administered conjugates can deplete B cells in an in vivo model

Having established that cells pre-coated with retargeting constructs can be killed in vivo, we investigated whether systemically injected conjugates effective using a hCD20 Tg mouse model frequently used evaluating anti-CD20 therapies [21]. These mice express hCD20 on all of their B cells, so depletion of circulating B cells can be used as measure of target cell killing. In vivo effectors were induced after OT-I transfer and immunisation. At the peak of the OT-I response (day 6), the retargeting constructs were administered i.v. and peripheral blood B-cell numbers at various time points determined. Figure 6a, b shows the time course of B-cell depletion for different doses of the F2 and F3 constructs, with the B-cell number expressed as a percentage of the pre-treatment value. Both F2 and F3 are able to deplete CD20⁺ B cells in vivo in a dose-dependent manner, with F3 being most effective and achieving up to 97 % depletion of circulating B cells for at least 7 days at a 1 nmole dose, almost matching the performance of 0.5 nmole of BsAb (Fig. 6c). To show that this B-cell depletion was due to the specific redirection of OT-I cells, experiments were repeated in WT mice that had received OT-I cells and in hCD20 Tg mice without OT-I transfer. The results confirmed that both hCD20 Tg targets and specific OT-I effectors were necessary for B-cell depletion (Fig. 6d).

Although our data demonstrate that the F2 and F3 conjugates are able to retarget CTL to kill hCD20⁺ targets both in vitro and in vivo and may also be able to induce CTL proliferation, the [anti-CD3 × anti-hCD20] BsAb still appears more effective across a range of assays. However, BsAb derivatives have been difficult to use in the clinic largely due to toxicity following systemic release of cytokines. We therefore compared the levels of inflammatory cytokines in the serum following 1 nmole of F3 and 0.5 nmole BsAb (doses chosen for their equivalent efficacy) in the B-cell depletion experiments described above (Fig. 6e). Seven of the nine cytokines assayed, IL-2, IFN- γ , TNF- α , IL-4, IL-5, IL-10, and KC, were present in the serum at a higher concentration after BsAb administration than after the F3 conjugate. Only IL-12 showed a higher serum concentration with F3 compared with BsAb.

Systemically administered conjugates are therapeutic in a tumour model

We also assessed the impact of conjugate administration on tumour growth in vivo. Mice were immunised with OVA and anti-CD40 to induce an endogenous anti-OVA CTL response, injected i.v with EL4 hCD20 cells (2×10^6) on day 3, then with retargeting conjugate (50 μ g) i.v on day 6 and i.p. on days 7, 8, and 9 (Fig. 7). Immunisation alone resulted in a significant increase in survival compared with no treatment, presumably because the anti-CD40 mAb is able to induce a weak anti-tumour response. However, both the F2 and F3 conjugates resulted in a clear survival advantage over immunisation alone, with the F3 being most effective with 6 of the 10 mice surviving beyond 100 days compared

with just one with immunisation alone. The effect of the BsAb was similar to that of the F2 conjugate, probably reflecting a lack of activated T cells in the naïve animals.

Discussion

We have investigated the retargeting of an OVA-specific CD8⁺ CTL population using a conjugate comprised of a mAb Fab' fragment and a single-chain MHC-peptide. This is an attractive alternative to anti-CD3-based BsAb which are indiscriminate in terms of the specificity of T cells that are recruited.

The earliest report of retargeting viral peptide-specific CTL to tumour cells was by Ogg et al. They used a bio-tinytated anti-CD20 mAb linked via an avidin bridge to biotinylated HLA-A2 monomers presenting an HIV gag-derived peptide to coat CD20⁺ tumour [29] and showed lysis of the CD20⁺ targets by gag-specific CTL. This unwieldy three-step process was refined by the same investigators to a two-step process using a tetrameric complex consisting of anti-CD20 scFv molecules genetically fused to streptavidin (B9E9SA) which could then combine directly with biotinylated pMHC. In an in vitro pre-clinical model of CLL, B9E9SA combined with biotinylated MHC presenting CMV peptide was able to redirect a patient's own ex vivo expanded CMV-peptide-specific CTL against their malignant CD20⁺ B cells [30]. This two-step retargeting system has also been evaluated in in vivo models in SCID and immunocompetent mice [31, 32]. The complexes were also able to cause in vivo expansion of the recruited CTL [31]. A similar two-step retargeting method was developed by Robert et al. [33] using Fab molecules rather than ScFv to target flu-specific CTL to CEA, ErbB-2 receptor, and CD20 on tumour cells.

While certainly proving proof-of-principle that CTL can be retargeted to malignant cells, this streptavidin-based system has limitations: it involves a large multivalent molecule which may have suboptimal tissue penetrance compared with a smaller single molecule. However, of greater concern is the immunogenicity of the biotin-streptavidin complex. Trials of B9E9SA to deliver ⁹⁰yttrium for treatment of metastatic colon cancer [34] and non-Hodgkin's lymphoma [35] have shown that the development of antibodies against the various components is almost inevitable. To circumvent these problems, various single-molecule derivatives have been designed. Robert et al. [36] refined their system into a one molecule consisting of a Fab fragment conjugated to a pMHC monomer. In vitro, this derivative was able to redirect flu-peptide-specific CTL to lyse tumour cells expressing CD20, ErbB-2, or CEA. These results were confirmed in vivo in an OT-I model using H2Kb/SIINFEKL peptide complex coupled to an anti-CEA Fab fragment [28] and also using endogenously generated anti-viral-peptide-specific CTL during an ongoing viral response [37]. Our chemically linked [SCT × Fab'] conjugates should also be considerably less immunogenic than streptavidin-based retargeting methods, while still allowing efficient killing of tumour cells by non-tumour-specific CTL.

We show that low concentrations of [SCT × Fab'] conjugates are able to coat CD20⁺ target cells, rendering them positive for H-2K^b-SIINFEKL as detected by binding of 25D1 antibody. These [SCT × Fab']-coated cells caused stimulation and proliferation of SIINFEKL-specific CTL in vitro and were also susceptible to lysis by low numbers of endogenously raised effector cells, both in vitro and in vivo. Injected complexes were also able to retarget SI-INFEKL-specific CTL to cause systemic B-cell depletion in OT-I recipient hCD20 transgenic mice and were also effective therapeutically in an EL4 tumour model.

An additional advantage which we have employed is the covalent linkage of peptide to the MHC molecule in the form of a SCT, leading to a greater stability of the reagent [19, 25]. In

soluble form, [SCT × Fab'] conjugates were unable to stimulate OT-I T cells, but had to be immobilised on CD20⁺ cell surface in order to crosslink the OT-I TCR. This is in comparison with conventional pMHC monomers, which can cause non-specific activation of T cells due to disassociation of peptide and loading onto MHC I on recipient cell surfaces [38]. The use of SCT incorporating a covalently linked peptide therefore also lowers non-specific activation of T cells. Covalently linked pMHC constructs have been previously used to retarget CTL to human xenographs in nude mice [39], and we have now shown their use to induce tumour cell killing by both adopted and endogenously raised CTL in immune competent animals.

[SCT × Fab'] retargeting constructs were able to cause killing of cells expressing both high (EL4h20) and low (B16h20) levels of huCD20. The sensitivity of TCR-mediated recognition of target is far higher than that of FcR-mediated activation [40, 41], and thus, this type of construct could be a suitable method for targeting tumour antigen expressed at low surface levels. The use of [SCT × Fab'] conjugates to recruit cells to kill tumour also represents a highly selective retargeting method allowing recruitment of MHCI-restricted T cells specific to the chosen peptide, compared with anti-CD3-based BsAb which allow activation of both CD4⁺ and CD8⁺ T cells in a nonspecific manner. Support for the potentially lower toxicity of the [SCT × Fab'] reagent compared with [anti-CD3 × anti-hCD20] BsAb can be found in the serum cytokine profiles of the hCD20Tg mice undergoing B-cell depletion; mice treated with the [SCT × Fab'] conjugates had lower levels of seven of the nine cytokines when compared with BsAb.

By removing the need to overcome tolerance against self/tumour antigens in order to raise a de novo response, retargeting of pre-existing non-tumour-specific CTL memory cells could represent an improved strategy for attacking tumours. While this investigation uses the model OVA antigen, immunological memory against microbial antigens has been used to provide T-cell help in anti-tumour vaccinations in mouse models [41], and similarly, vaccination of cancer patients with microbial antigen could result in the expansion of high-affinity memory cells providing a potential pool of CTL which could be retargeted to tumour. Compared with ex vivo expansion of tumour-specific or CAR-expressing CTL, the delivery of [SCT × Fab'] conjugates to retarget anti-viral CTL boosted by vaccination could be a more convenient and cost-effective process, requiring no ex vivo cell culture, and simpler storage and administration.

This proof-of-concept study shows that it is possible to retarget non-tumour-specific CTL to kill cancer cells by use of [SCT × Fab'] reagents. By designing reagents to retarget a selected pool of efficient, high-affinity anti-viral effector CTL, this strategy has the potential to be as efficacious as bispecific anti-CD3 reagents, but, by avoiding the activation of a large number of T cells, should show more limited toxicity. Further studies into the efficacy and toxicity of SCT-based retargeting reagents are therefore required.

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Fig. 1.

Production of the SCT. **a** DNA construct for the bacterial expression of SCT. **b** SDS-PAGE showing purification of SCT from inclusion bodies: *lane 1*, whole bacterial lysate (sonicated); *lane 2*, supernatant from ultracentrifuged lysate (soluble fraction); *lane 3*, washed and solubilised inclusion bodies. **c** Splenocytes from wild-type and OT-I mice were labelled with tetramers (5 $\mu\text{g/ml}$) formed from biotinylated SCT and PE-streptavidin and APC-anti-CD8. The *upper plot* shows that these tetramers bind specifically to CD8⁺ T cells from OT-I mice. The *lower plot* shows the binding obtained with a commercial tetramer for comparison

Fig. 2.

Production and binding activity of [SCT × Fab'] conjugates. SCT and anti-hCD20 (AT80) Fab' were conjugated using SMCC as described in "Materials and methods". **a** SDS-PAGE analysis of SMCC conjugation products: *lanes 2–5*, non-reduced; *lanes 7–10*, reduced. *Lanes 1 and 6*, mol wt markers; *lanes 2 and 7*, AT80 F(ab')₂; *lanes 3 and 8*, F1 conjugation fraction; *lanes 4 and 9*, F2 conjugation products; *lanes 5 and 10*, F3 conjugation products. The *arrows* show the position of AT80 Fab' H chain and L chain. **b** Binding of [SCT × anti-CD20] conjugates to Daudi cells. F2 or F3 conjugates were added to Daudi and then bound conjugate detected using FITC-25D1. *Open symbol* shows result for Daudi cells pre-incubated with excess AT80 IgG before the addition of F2 or F3 conjugate. The *horizontal dashed line* represents FITC-25D1 staining of EG7 cells. One of two similar experiments

Fig. 3.

[SCT × Fab'] conjugates can redirect OVA-specific CTL to kill tumour cells in vitro. The ability of the F2 and F3 conjugates to redirect non-cognate CTL to kill tumour cells was assessed in vitro using a ^{51}Cr release assay. Splenocytes from immunised OT-I recipient mice were used as a source of activated effector CTL (Materials and methods). **a** CTL effectors were incubated with ^{51}Cr -labelled Daudi cell targets in the presence of F2 or F3 conjugates or an [anti-CD3 × anti-hCD20] BsAb and ^{51}Cr release determined after 4 h; C57BL/6 splenocytes were used as irrelevant effectors. **b** The assay was repeated (1 µg/ml conjugate) in the presence of excess anti-H-2k^b/SIINFEKL mAb (25D1; blocks conjugate:effector) mAb or anti-CD20 F(ab')₂(blocks conjugate:target) (10 µg/ml). The effector target ratio was 10:1 throughout

Fig. 4.

Cell-bound conjugates can induce proliferation of the targeted CTL population in vitro. **a** Purified OT-I T cells were incubated with 1 μ g/ml of F2 or F3 conjugate or [anti-CD3 $\times$ anti-hCD20] BsAb in the presence or absence of 1×10^4 (lo) or 1×10^5 (hi) irradiated CD20⁺ Daudi cells. EG7 cells (express H-2K^b-SIINFEKL) were included as a positive control. 1 μ Ci [³H] thymidine was added per well for the final 18 h of a 72 h incubation, cells were then harvested and incorporation measured. *Error bars* represent the mean and SEM from triplicate wells and the results are representative of several experiments. **b** Representative histograms from a CFSE dilution experiments. Similar conditions were used to those in **a** except OT-I cells were labelled with 2 μ M CFSE prior to incubation. After 72 h, cells were labelled with APC-anti-mouse CD8, and CFSE dilution was determined using a FACSCalibur. Results are representative of several experiments

Fig. 5.

[SCT × Fab'] conjugates can target an OVA-specific CTL to kill target cells in vivo. **a** and **c** Splenocytes from OT-I mice were transferred into C57BL/6 recipients and these were then immunised with anti-CD40 mAb and OVA to induce a population of in vivo effector cells (Materials and methods). 5 days later, these mice were used as immunised OT-I recipients; at this time, approximately 15 % of total splenocytes were OT-I CTL. **b** and **d** C57BL/6 mice were immunized with anti-CD40 mAb and OVA to induce a population of endogenous effectors (Materials and methods). 6 days later, these mice were used as immunized recipients; at this time, approximately 1–2 % of total splenocytes were SIINFEKL-specific. **a** hCD20 Tg (target) or non-Tg (non-target) C57BL/6 mouse splenocytes were labelled with high or low levels of CFSE respectively, mixed (1:1), incubated with conjugate (5 µg/ml) and injected i.v. into naïve or immunized OT-I recipient mice. 20 h later, splenocytes were labelled with APC-anti-CD19 and assessed by flow cytometry for the relative proportion of surviving hCD20 Tg B cells, expressed as the target:non-target ratio (T:NT). The main figure (*left*) shows the mean and range for two mice; 1 of 2 similar experiments. The plots (*right*) show representative data from immunized recipients receiving F1- and F3-coated target cells. **b** as **a**, but with transfer into recipients with endogenous effectors (induced as described above). **c** EL4h20 cells (target, T) labelled with CFSE and PKH26 and EL4 cells (non-target, NT) labelled with CFSE alone were mixed (1:1), incubated with conjugate (0.2, 2 or 20 µg/ml) and injected i.p. into naïve or immunized OT-I recipient mice. 20 h later, peritoneal cavity cells were assessed by flow cytometry for the relative proportion of surviving EL4h20 cells (T:NT ratio). The main figure (*left*) shows the mean and range for two mice; 1 of 2 similar experiments. The inset plots show the expression of CD20 on the EL4h20 cells, and data from naïve (*top*) and immunized (*bottom*) recipients receiving F3 (20 µg/ml)-coated target cells. **d** B16h20 cells were incubated with 5 µg/ml conjugate and then injected i.v. into naïve mice or mice with endogenous effectors (induced as described above). After 28 days, mice were killed and the number of pigmented melanoma lung colonies counted. The *plot* shows mean and range from three mice. *p* values were calculated using a *t* test. The inset shows the expression of CD20 on the B16h20 cells

Fig. 6.

Time course of B-cell depletion in a hCD20 Tg model with F2 and F3 conjugates. In vivo effectors were induced by transferring OT-I cells into hCD20 Tg recipient mice and immunising with OVA and anti-CD40 as described in Fig 5. At the peak of the OT-I activation (day 6), the retargeting constructs were administered i.v. at the doses shown and peripheral blood B-cell numbers at various time points were determined by flow cytometry. **a** F2 conjugate; **b** F3 conjugate; **c** [anti-CD3 $\times$ anti-hCD20] BsAb. The number of B cells is expressed as a % of the pre-treatment value, and points represent mean $\pm$ SD ($n = 4$, results combined from 2 separate experiments). **d** *Left panel* OT-I cells were transferred into wild-type mice which were then immunised and receive F2 and F3 conjugates and BsAb as described above. *Right panel*: hCD20 Tg mice were immunised in the absence of OT-I cells. Points show mean and range from 2 mice. **e** Serum cytokine levels were measured 2 h after i.v. injection of 1 nmole F3 or 0.5 nmole bispecific F(ab')₂ using the Meso Scale Discovery TH1/TH2 9-plex kit. The bars show the mean $\pm$ range

Fig. 7.

Therapeutic activity of F2 and F3 conjugates in an EL4h20 model. C57BL/6 mice (10/group) were immunised i.v. with 5 mg OVA and 0.5 mg anti-CD40 mAb on day 0. On day 3 mice received 2×10^6 EL4hu20 cells i.v., then treated on day 6 with 50 μ g retargeting conjugate or BsAb i.v., followed by further 50 μ g i.p. injections on days 7, 8 and 9. Control groups included naïve mice, untreated immunised mice, and naïve mice treated with BsAb. Results were analysed using a Log-rank (Mantel Cox test). Naïve versus immunised, $p < 0.05$; immunised versus immunised + F3, $p < 0.01$; immunised versus immunised + F2, $p = 0.05$