Selective Expansion of Chimeric Antigen Receptor-targeted T-cells with Potent Effector Function using Interleukin-4∗

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Polyclonal T-cells can be directed against cancer using transmembrane fusion molecules known as chimeric antigen receptors (CARs). Although preclinical studies have provided encouragement, pioneering clinical trials using CAR-based immunotherapy have been disappointing. Key obstacles are the need for robust expansion ex vivo followed by sustained survival of infused T-cells in patients. To address this, we have developed a system to achieve selective proliferation of CAR+ T-cells using IL-4, a cytokine with several pathophysiologic and therapeutic links to cancer. A chimeric cytokine receptor (4αβ) was engineered by fusion of the IL-4 receptor α (IL-4Ra) ectodomain to the β subunit, used by IL-2 and IL-15. Addition of IL-4 to T-cells that express 4αβ resulted in STAT3/STAT5/ERK phosphorylation and exponential proliferation, mimicking the actions of IL-2. Using receptor-selective IL-4 muteins, partnering of 4αβ with γc was implicated in signal delivery. Next, human T-cells were engineered to co-express 4αβ with a CAR specific for tumor-associated MUC1. These T-cells exhibited an unprecedented capacity to elicit repeated destruction of MUC1-expressing tumor cultures and expanded through several logs in vitro. Despite prolonged culture in IL-4, T-cells retained specificity for target antigen, type 1 polarity, and cytokine dependence. Similar findings were observed using CARs directed against two additional tumor-associated targets, demonstrating generality of application. Furthermore, this system allows rapid ex vivo expansion and enrichment of engineered T-cells from small blood volumes, under GMP-compliant conditions. Together, these findings provide proof of principle for the development of IL-4-enhanced T-cell immunotherapy of cancer.

Adoptive T-cell immunotherapy is an exciting platform technology pioneered to treat hematologic malignancies and metastatic melanoma. To broaden applicability, T-cell specificity may be targeted against diverse cancers using genetically delivered chimeric antigen receptors (CARs). These fusion receptors couple the binding of a native tumor-associated target molecule to the delivery of a tailored T-cell activating signal. Several CARs have been described that mediate potent antitumor activity in vitro (1–3) and in preclinical in vivo models (4–8). Disappointingly, however, CAR-based immunotherapy has only achieved modest results for patients. A key impediment to success has been poor T-cell survival following infusion (9, 10). Measures that improve this, for example CAR delivery to virus-specific T-cells, have yielded evidence of improved efficacy (11).

Survival and growth of T-cells depend critically upon cytokines. The most potent of these for effector T-cells are IL-2 and IL-15, both of which signal using common β and γc receptor subunits. IL-2 is licensed for administration to patients with some selected tumor types. However, systemic IL-2 stimulates lymphocytes in an indiscriminate manner and is frequently profoundly toxic (12). To refine this, we have developed a system whereby an IL-2/15-like signal can be selectively provided using IL-4, a cytokine with much weaker intrinsic mitogenic activity for T-cells (13).

IL-4 was chosen to provide this selective growth signal for two additional reasons. First, “endogenous” IL-4 plays a key albeit complex role in several tumor types. Second, administration of “exogenous” IL-4 has been tested clinically as a therapeutic option for metastatic cancer in man (see below).

Endogenous IL-4 may be elaborated by cancer cells or by infiltrating leukocytes and exerts pleiotropic but variable actions upon the cellular components found in established tumors. First, IL-4 increases the proliferation of some cancer-derived cell lines (14, 15), protects tumor cells from apoptosis (16–18), and promotes the propagation of colorectal cancer stem cells (19). Second, IL-4 supports (prometastatic) M2-polarized macrophages (20, 21) and may achieve several suppressive effects on tumor-specific T-cells (22). On the other hand, IL-4 also inhibits the growth of a range of other cancer cell types.

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(23, 24). Furthermore, IL-4 may antagonize tumor progression through local recruitment of neutrophils and eosinophils (25, 26), dysregulation of stromal fibroblasts (27), and inhibition of angiogenesis (28). Reflecting this complexity, type 2 polarized tumor-infiltrating T-cells may either promote tumor rejection (22, 29) or progression (20–22), depending upon model system.

Despite the intricate relationship between endogenous IL-4 and cancer, administration of supraphysiologic amounts of this cytokine has been widely tested as an experimental therapy for several malignancies. Preclinical investigators have repeatedly demonstrated potent antitumor activity of exogenous IL-4 in several in vivo models (25, 26, 30, 31). Although efficacy in clinical studies has been limited, these studies have allowed the identification of safe ceiling doses in man (32, 33). Together, this experience provides a platform for development of further immunotherapeutic approaches that involve the administration or harnessing of this cytokine.

IL-4 interacts with two heterodimeric receptors. Both share a high affinity α subunit (IL-4Ra), which pairs with either γc (type 1 receptor) or IL-13Ra1 (type 2). Activated T-cells express the type 1 receptor, allowing IL-4 to support their survival and promote limited proliferation (13). To harness IL-4 binding to delivery of a potent mitogenic signal for T-cells, the ectodomain of IL-4Ra has been fused to the endodomain of the shared IL-2/15 β subunit, thereby creating 4αβ (34, 35). Here, we show that IL-4 can be used to drive the selective expansion and sustained antitumor activity of T-cells in which a tumor-specific CAR is co-expressed with 4αβ.

EXPERIMENTAL PROCEDURES

Recombinant DNA Constructs—In 4αβ, cDNAs encoding the IL-4Ra ectodomain (amino acids 1–233) were fused to the transmembrane and endodomain of βc (amino acids 241–551; see Fig. 1A). Gene synthesis was performed by GenScript (Piscataway, NJ). The chimeric cDNA was inserted into the SFG retroviral vector (2, 6, 7), upstream of indicated CARs. Inserts were separated by a furin cleavage site and Thseas asigna (T2A) peptide (36) (Fig. 1). 4αβ was co-expressed with the following CARs: (i) HOX, specific for MUC1 (7); (ii) HTr, a matched endodomain truncated control (7) (Fig. 1); (iii) P28z, specific for prostate-specific membrane antigen (PSMA); (iv) PTr, a matched endodomain truncated control; and (v) ErbB-28z, targeted against ErbB receptor tyrosine kinases (to be described fully in a forthcoming publication). Additional control CARs were: (i) DOX, which lacks an scFv but is otherwise identical to HOX (7) and (ii) C28z, which is identical to P28z but contains a humanized PR1A3-derived scFv, specific for carcinoembryonic antigen.

Cytokines—Human IL-2 (aldesleukin) was from Novartis (Frimley, UK). Human IL-4 was from Peprotech (London, UK) and, in GMP grade, from Gentaur (Brussels, Belgium). The Histagged IL-4 mutesins R121D Y124D (IL-4DD) (37) and T13D R121E (IL-4DE) (38) were expressed and purified from Escherichia coli by GenScript.

Cell Lines—All tumor cell lines were obtained from authenticated stocks provided by the Cancer Research UK organization. PG13 retroviral packaging cells were obtained from the European Collection of Cell Cultures and were maintained in Dulbecco’s modified Eagle’s medium + 10% fetal calf serum. H29D retroviral packaging cells were a gift from Dr. Michel Sadelain (Memorial Sloan Kettering Cancer Center, New York) and were propagated as described (2). CTLL-2 is a murine IL-2-dependent CD8+ T-cell line (gift from Dr. Alastair Noble, King’s College London) and was maintained as described (39). Retroviral transduction of CTLL-2 cells was achieved using supernantant derived from H29D retroviral packaging cells (2).

Cytokines—Human IL-4 was from Peprotech (London, UK) and was maintained as described (39). Intracellular staining was performed following activation with PMA and ionomycin for 6 h, as described (40).

Western Blotting—Western blotting for γ chain-containing fusion receptors (2, 7) and ERK phosphorylation (39) was performed as described. Immunoblotting of HSC 70 was performed using the B6 mouse monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Detection of phosphorylated and total STAT3, 5, and 6 was performed using antibodies from Cell Signaling Technology (Danvers, MA).

Upscaled Production of Genetically Engineered T-cells—Freshly isolated peripheral blood mononuclear cells (3 × 10^6 cells/ml) were cultured in a 100-ml Cell Expansion Bag (Miltenyi Biotec, Bergisch Gladbach, Germany) in AIM V medium (Invitrogen) containing paramagnetic anti-CD3/anti-CD28 Dynabeads (Invitrogen; 1 bead/cell). After 48 h, 100 units/ml IL-2 was added. After 72 h, retroviral transduction was performed using a VueLife™ adherent FEP bag (187 cm^2; American Fluoroseal Corporation, Gaithersburg, MD). The bag was precoated overnight at 4 °C with retronectin (5 μg/cm^2; Takara, Shiga, Japan), which was replaced with PG13-derived vector (50 ml). Activated peripheral blood mononuclear cells (20 × 10^6 in IL-2 (100 units/ml) were added after 1 h without centrifugation. Starting after 24 h, IL-4 (30 ng/ml) was added with each medium addition. Day 3 after transduction, T-cells were expanded in 250-ml Cell Differentiation bags (Miltenyi Biotec).

Cytotoxicity Assays—Four-hour CTL assays were performed using a lactate dehydrogenase release assay (Roche Applied Science) (2, 40). Tumor monolayers were visualized using crystal violet (0.5% in 25% methanol) after fixation with ice-cold methanol for 10 min.

Statistical Analysis—The one-tailed Student’s t test was used.

RESULTS

4αβ Chimeric Cytokine Receptor Confers IL-4 Responsiveness upon CTLL-2 Cells—CTLL-2 cells are absolutely dependent upon IL-2 or IL-15 but are unresponsive to human IL-4. Consequently, these cells provide an excellent model to test the ability of 4αβ to deliver an IL-2/15-like signal in response to

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human IL-4. 4αβ was co-expressed in CTLL-2 cells with the MUC1-specific CAR, HOX (4αβ-HOX) or truncated control, HTr (4αβ-HTr) (Fig. 1). Cell surface expression of 4αβ was demonstrated by flow cytometry (Fig. 2A). To assess function, cells were stimulated using human IL-2 or IL-4. In 4αβ-expressing cells, both STAT5 and p42/44 ERKs underwent comparable phosphorylation in response to either cytokine. However, HOX+ or parental CTLL-2 cells only responded to IL-2 in these assays (Fig. 2B).

We next compared the ability of human IL-2 and IL-4 to sustain proliferation of CTLL-2 cells. Both IL-2 and IL-4 elicited dose-dependent expansion of cells that express 4αβ (Fig. 2C). By contrast, IL-2 but not IL-4 could stimulate proliferation of parental or HOX+ cells. We anticipated that 4αβ mediates responsiveness to IL-4 following heterodimerization with γc. To confirm this, assays were also performed using two human IL-4 muteins. The T13D R121E mutein (IL-4DE) is an agonist of the type 1 but not type 2 IL-4 receptor (38) and also promoted phosphorylation of STAT5 whereas IL-4 was weaker in this respect. As expected, phosphorylation of STAT5 in engineered primary T-cells was potent. Phosphorylation of STAT3 and p42/44 ERKs was assayed by Western blotting. Cell numbers were reevaluated after 6 days. Similar results were obtained in two to five independent experiments.

Expression of IL-4Ra/β, in Tumor-targeted Human T-cells—Next, human T-cells were engineered to co-express 4αβ-HOX or 4αβ-HTr. A representative flow cytometric analysis is shown in Fig. 3A. Stoichiometric co-expression of chimeric antigen and cytokine receptors was achieved using an intervening T2A peptide placed downstream of a furin cleavage site (Fig. 1B). To exclude inefficient ribosomal skipping using this system, Western blotting was performed (note absence of uncleaved precursor at top of 4αβ-HOX lane; Fig. 3B). We were unable to source a commercial IL-4Ra antibody that permitted detection of IL-4Ra or 4αβ by Western blotting, despite multiple attempts using antibodies marketed for this purpose (data not shown).

To investigate signaling by 4αβ in human T-cells, phosphorylation of STATs 3, 5, and 6 were examined by Western blotting (Fig. 3C). Comparison was made among the actions of IL-2, IL-4, and IL-15 on T-cells that express HOX alone or 4αβ-HOX. In control T-cells, IL-2 and IL-15 elicited strong phosphorylation of STAT5 whereas IL-4 was weaker in this respect. Phosphorylation of STAT3 in engineered primary T-cells was very weakly evident in response to any cytokine. By contrast, in 4αβ-HOX+ T-cells, we observed potent phosphorylation of both STAT3 and STAT5 in response to IL-4. As expected, phosphorylation of STAT6 was also induced by IL-4 in all T-cells (Fig. 3C). Despite using freshly made reducing agents, we also consistently detected a second specific band in phos-
Interleukin-4 Supports Tumor Cell Cytotoxicity by 4αβ-expressing T-cells—Previously, we have reported that HOX enables human T-cells to elicit MUC1-dependent destruction of several tumor monolayers, accompanied by production of IFN-γ (7). Here, we observed that neither monolayer destruction (supplemental Fig. S1) or production of IFN-γ by HOX-expressing T-cells (supplemental Fig. S2A) was altered by co-expression of 4αβ. Similar results were observed when 4αβ was co-expressed with the PSMA-specific CAR, P28z (supplemental Fig. S2B8 and data not shown). As expected, control 4αβ-HTr-expressing T-cells were inactive in these assays (supplemental Figs. S1 and S2A).

Several reports have indicated that culture in IL-4 can impair T-cell cytotoxicity (22), which would clearly be undesirable for tumor immunotherapy. To examine tumor cell killing, T-cells were engineered to express 4αβ-HOX or 4αβ-HTr, propagated in IL-2 or IL-4 for 1 week and then established in cytotoxicity assays. Using tumor cell targets that were discordant for MUC1 expression, we observed that HOX+ T-cells elicited MUC1-dependent cytotoxicity in 4-h assays, as previously described (7; Fig. 4B). However, tumor cell destruction was clearly impaired by pregrowth in IL-4 (Fig. 4B). This was more apparent in overnight assays in which equal numbers of T-cells and tumor cells were co-cultivated (Fig. 4C). By contrast, 4αβ-HOX+ T-cells elicited comparable tumor cell destruction, whether precultured in IL-2 or IL-4 (Fig. 4, B and C). We also observed that 4αβ-HOX T-cells underwent greater enrichment when cultured in IL-4 than IL-2 (compare Fig. 4, A and C).

Interleukin-4 Can Support Long-term Expansion and Repeated Tumor Cell Destruction by 4αβ-HOX+ T-cells—Next we examined the ability of IL-4 to maintain proliferation and antitumor activity of MUC1-targeted and control T-cells that co-express 4αβ. Fig. 5 shows long-term culture experiments in which engineered T-cells were cultured with three MUC1+ tumor cell lines. As described previously (7), T-cells that express HOX elicited repeated tumor cell destruction when cultured with IL-2. However, rapid loss of antitumor activity was observed when HOX+ T-cells were cultured with IL-4. Control 4αβ-HTr+ T-cells did not elicit any meaningful anti-tumor activity, irrespective of whether they were maintained in IL-2 or IL-4. By contrast, 4αβ-HOX+ T-cells repeatedly destroyed tumor monolayers and underwent exponential T-cell expansion. The magnitude of T-cell expansion and the
number of tumor restimulation cycles achieved were consistently greater when 4αβ-HOX+ T-cells were cultured in IL-4 than IL-2. Indeed, 4αβ-HOX+ T-cells expanded up to 7 logs when repeatedly exposed to tumor targets (e.g. MUC1-transfected MDA-MB-435 cells (Fig. 5C). Comparable T-cell expansion was achieved using IL-4 or the hematopoietic-selective IL-4DE mutein (supplemental Fig. S3), in agreement with data obtained using CTL2-2 cells (Fig. 2C). Similar results were obtained when 4αβ-HOX T-cells were cultured with two other MUC1+ tumor cell lines, ZR75 and MCF7 (supplemental Fig. S4, A and B, respectively). Importantly, following culture in the sustained presence of IL-4, the cytolytic activity of 4αβ-HOX+ T-cells remained target antigen-specific (Fig. 5D). Furthermore, T-cells that co-express 4αβ-HOX remained absolutely dependent upon exogenous IL-4 for sustained expansion. When deprived of IL-4, increased apoptotic cell death was observed within 24 h (supplemental Fig. S5), leading to loss of ≥95% viable cells within 10 days.

To assess generality of application, 4αβ has also been co-expressed with a CAR targeted against PSMA (P28z) (2). When 4αβ-P28z T-cells were repeatedly cultured with PSMA-expressing LNCaP tumor cells, T-cell expansion was greater in the presence of IL-4 than IL-2. By contrast, the converse was the case for T-cells that express P28z alone (supplemental Fig. S4C and data not shown).

Use of IL-4 to Achieve Rapid ex Vivo Expansion of Genetically Engineered T-cells—We have also co-expressed 4αβ with a CAR that targets ErbB receptor tyrosine kinases. Experiments were designed to develop a clinically robust process to expand T-cells rapidly ex vivo using IL-4, for intratumoral therapy of locally recurrent head and neck cancer. Fig. 6 demonstrates that IL-4 promotes exponential expansion (Fig. 6A) accompanied by selective enrichment (Fig. 6B) of ErbB targeted T-cells over a 10-day period. By contrast, cells expanded in IL-2 did not undergo enrichment during ex vivo expansion (Fig. 6C). Similar findings were observed using the PSMA-specific CAR, P28z (supplemental Fig. S6) and the MUC1 CAR, HOX (data not shown). Cultures supplemented with IL-4 exhibited a type 1 polarized phenotype. This was indicated by intracellular staining for marker cytokines (Fig. 6D) and by production of large quantities of IFN-γ following tumor monolayer stimulation, irrespective of the presence or absence of IL-4 (data not shown). Engineered T-cells remained dependent upon provision of exogenous cytokines; in the absence of CAR stimulation, they underwent apoptotic cell death when deprived of exogenous IL-4 (supplemental Fig. S7). Functional studies demonstrated potent tumor destructive activity against a panel of ErbB-expressing tumor cell lines in vitro and following adoptive transfer to mice bearing established tumor xenografts. Immunophenotyping demonstrated that IL-4-expanded T-cells expressed a predominantly effector memory phenotype with high levels of NKG2D (supplemental Fig. S8).

DISCUSSION

Survival of tumor-specific T-cells following adoptive transfer has been correlated with successful T-cell immunotherapy of cancer (42). In this study, we describe a genetic approach to achieve regulated T-cell expansion and survival using IL-4. We have exploited the use of a chimeric cytokine receptor (4αβ)
IL-4-responsive T-lymphocytes

that couples the binding of IL-4 to achieve sustained T-cell expansion and tumor cytolytic activity. The capacity of an IL-4R-selective mutein (IL-4DE) (38) to act similarly is consistent with a model in which IL-4 promotes heterodimerization of 4αβ with γc to activate signaling pathways used by IL-2 and IL-15. T-cells that express 4αβ expand exponentially in response to IL-4 over a cycle of 7–10 days, during which period they also undergo selective enrichment. If periodically reactivated via a tumor-specific CAR, T-cells exhibit sustained and durable tumor-destructive activity and can expand through several logs in vitro. Importantly, cytolytic activity of such T-cells remains specific for tumor antigen. Furthermore, although IL-4 signaling remains intact (indicated by STAT6 phosphorylation), 4αβ+ T-cells do not undergo type 2 polarization.

An important and unanticipated finding of our study was the superiority of IL-4 over IL-2 in supporting the expansion of 4αβ+ T-cells at the concentrations selected. This cannot be ascribed to the ability of IL-4 to enrich for 4αβ-CAR-grafted T-cells because repeated culture on tumor monolayers in IL-2 also achieves this (2, 7). A more probable explanation is that more enhanced signaling potency is delivered by IL-4 via 4αβ/γc than is provided by IL-2 or IL-15 via β/γc. Phosphorylation of STAT3 was weakly observed in engineered T-cells in response to IL-2 or IL-15 and is known to depend upon preexisting activation state (43). By contrast, IL-4 achieved potent STAT3 phosphorylation in T-cells that co-express 4αβ with HOX. The high affinity of IL-4Ra for IL-4 coupled with the constitutive expression of 4αβ in engineered cells may account for this, in particular because γc is up-regulated rapidly upon T-cell activation (44). Alternatively, this may reflect an unanticipated role for the CAR endodomain because we did not observe STAT3 phosphorylation when 4αβ was co-expressed with the truncated CAR, HTr (data not shown).

IL-4 represents something of a “double edged sword” as a cancer immunotherapeutic agent because it elicits a panoply of both antitumor and protumor activities (see Introduction). Three key attributes of this cytokine have been cited as hindering tumor immunity, namely reduction of CD8+ T-cell number, polarization to a type 2 phenotype, and reduced cytolytic activity (22). However, none of these caveats applies to the use of IL-4 to expand 4αβ-grafted T-cells. The lack of IL-4-induced type 2 polarization may reflect the distinct signals delivered via 4αβ coupled with the fact that T-cells are not exposed to IL-4 prior to initial activation. The potent cytolytic activity of these cells is consistent with the known capacity of IL-2/15 to enhance the effector function of cytotoxic T-cells. Although cytolytic activity remained antigen-specific, we are currently exploring the contribution of heightened NKG2D expression to this process (45).

Three clinical applications of this system can be envisioned. First, as demonstrated here, IL-4 can be used to achieve rapid and selective ex vivo expansion of CAR-engineered T-cells without complex equipment such as bioreactors. Use of this system will facilitate the robust generation of therapeutic cell products in the “real world” of patients with disease and therapy-related immune compromise. Second, use of T-cells engrafted with 4αβ may permit therapeutic exploitation of the elevated level of endogenous IL-4 associated with a variety of tumor types (16, 17, 19, 20, 46). Indeed, elevated circulating IL-4 has been advocated as a biomarker of the progression of certain malignancies (47, 48). We anticipate that 4αβ+ T-cells will acquire a selective benefit in such patients, particularly in the tumor microenvironment and are devising in vivo studies to test this hypothesis.

A third therapeutic opportunity raised by this study entails the administration of exogenous IL-4 to provide selective support for adoptively transferred T-cells in vivo. This is a particularly attractive option in diseases in which IL-4 exerts a direct antitumor effect, such as B-cell malignancy (33). To enhance efficacy further, the hematopoietic selective mutein, IL-4DE, may be used (38), in particular because it exerts greater activity than does IL-4 against transformed B-cells (49). Furthermore, because IL-4-mediated toxicity is believed to derive mainly from engagement of the type 2 IL-4R, the therapeutic index should be enhanced further. We have shown that IL-4DE promotes expansion of 4αβ-expressing T-cells that is comparable with IL-4. Consequently, it would be of interest to co-express 4αβ with a CD19 CAR in patients with refractory B-cell malignancy, using IL-4DE to attain controlled in vivo T-cell expansion and function.

Use of the hematopoietic selective IL-4DE mutein in preference to IL-4 may also be advantageous in development of adoptive immunotherapy for solid tumors. Because IL-4DE is a selective agonist of the type 1 IL-4 receptor, it would be expected to exert antagonistic actions on tumors where IL-4 achieves direct tumor-supportive effects (14–19). We are cur-
IL-4-responsive T-lymphocytes
currently developing in vivo models to test efficacy of CAR-grafted T-cells when supported by systemically administered IL-4 and derived muetins.

Finally, the availability of a complete IL-4 antagonist (IL-4DD) will facilitate the clinical development of this approach. Such an agent is under active development for refractory asthma (50), but could prove useful as a safety measure to effect rapid clearance of IL-4-dependent T-cells in vivo. Taken together, our data support the development of a clinically useful rapid clearance of IL-4-dependent T-cells in vivo. Taken together, our data support the development of a clinically useful rapid clearance of IL-4-dependent T-cells in vivo. Taken together, our data support the development of a clinically useful rapid clearance of IL-4-dependent T-cells in vivo. Taken together, our data support the development of a clinically useful rapid clearance of IL-4-dependent T-cells in vivo. Taken together, our data support the development of a clinically useful rapid clearance of IL-4-dependent T-cells in vivo. 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