RESEARCH ARTICLE

Overcoming the Immunosuppressive Tumor Microenvironment of Hodgkin Lymphoma Using Chimeric Antigen Receptor T Cells

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ABSTRACT

Patients with otherwise treatment-resistant Hodgkin lymphoma could benefit from chimeric antigen receptor T-cell (CART) therapy. However, Hodgkin lymphoma lacks CD19 and contains a highly immunosuppressive tumor microenvironment (TME). We hypothesized that in Hodgkin lymphoma, CART should target both malignant cells and the TME. We demonstrated CD123 on both Hodgkin lymphoma cells and TME, including tumor-associated macrophages (TAM). In vitro, Hodgkin lymphoma cells convert macrophages toward immunosuppressive TAMs that inhibit T-cell proliferation. In contrast, anti-CD123 CART recognized and killed TAMs, thus overcoming immunosuppression. Finally, we showed in immunodeficient mouse models that CART123 eradicated Hodgkin lymphoma and established long-term immune memory. A novel platform that targets malignant cells and the microenvironment may be needed to successfully treat malignancies with an immunosuppressive milieu.

SIGNIFICANCE: Anti-CD123 chimeric antigen receptor T cells target both the malignant cells and TAMs in Hodgkin lymphoma, thereby eliminating an important immunosuppressive component of the tumor microenvironment. Cancer Discov; 7(10); 1–14. ©2017 AACR.

INTRODUCTION

Despite multimodality therapy, a subset of patients with Hodgkin lymphoma succumbs to this disease. In particular, 10% to 15% of patients presenting with localized disease and 20% to 40% with advanced-stage disease will experience relapse (1). Furthermore, an additional 10% to 15% of patients with Hodgkin lymphoma have disease that is refractory to first-line therapy (2). Recent developments in biological therapy for relapsing/refractory (r/r) Hodgkin lymphoma include the anti-CD30 antibody–drug conjugate brentuximab vedotin (BV) and PD-1 inhibition (3). Although BV induces objective responses in 75% of patients with r/r Hodgkin lymphoma after autologous stem cell transplantation (SCT), responses are not durable and the median progression-free survival is only 5.6 months (4). Similarly, PD-1 inhibition leads to high overall response rates, but most of these are partial (5). Thus, despite the profusion of agents for Hodgkin lymphoma, there is still a need for therapies with curative potential.

The role of adoptive cellular therapy in Hodgkin lymphoma has been demonstrated by the effectiveness of allogeneic SCT in a subset of patients with Hodgkin lymphoma and, more recently, by the promising clinical results of the infusion of Epstein-Barr virus (EBV)–specific T cells (6). However, allogeneic SCT carries a high treatment-related mortality, and only approximately 30% to 40% of Hodgkin lymphoma expresses the EBV-encoded antigens (7, 8). Thus, T-cell–related therapies already display an important and growing role in Hodgkin lymphoma. Chimeric antigen receptor T cells (CART) represent an exciting recent development in cancer immunotherapy (9). Our group and others have demonstrated the clinical efficacy of anti-CD19 chimeric antigen receptor redirected T cells (CART19) for refractory B-cell malignancies (10–14). However, despite the B-cell origin of Hodgkin Reed-Sternberg (HRS) cells, B-cell antigens, including CD19, are rarely expressed in Hodgkin lymphoma (15). Although the CD30 antigen is known to be commonly expressed on HRS cells, outcomes of patients treated with anti-CD30 CART have been relatively disappointing, with reported 1 in 8 complete responses (CR), 4 in 8 stable disease (SD), and 3 in 8 progressive disease (PD) in patients with r/r Hodgkin lymphoma (16–18). In Hodgkin lymphoma, malignant cells represent only approximately 1% to 2% of cellularity, with the majority of the tumor microenvironment (TME) cells being comprised of infiltrating immune cells (macrophages and myeloid-derived suppressive cells, basophils, mast cells, eosinophils, B and T lymphocytes, stromal cells and fibroblasts). The TME and in particular tumor-associated macrophages (TAM) play a key role in promoting tumor growth while also inhibiting the antitumor immune response. High frequency of CD68+ TAMs in Hodgkin lymphoma biopsies has been correlated with poor overall survival (19–25). Immunosuppressive and protumor macrophages have been defined as M2 type, as opposed to inactivated (M0) or anti-infection/tumor macrophages (M1; refs. 26–28). Thus, Hodgkin lymphoma represents a unique opportunity to study the impact of the TME on immunotherapy. The development of an approach that could target the malignant cells as well as the supportive TME would likely represent an important advance in the field of CART immunotherapy, by providing robust stimulation of the CART cells while avoiding T-cell inhibition.

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In this context we studied CD123, the α chain of the receptor for IL3, whose expression has been previously described on Hodgkin HRS cells (29–33). In addition, in vitro data show that IL3 rescues Hodgkin lymphoma cells from apoptosis and promotes Hodgkin lymphoma cell line growth (34). Furthermore, because CD123 is expressed on myeloid cells, including macrophages, plasmacytoid dendritic cells, eosinophils, basophils, and mast cells, we hypothesized that CD123 would be expressed within Hodgkin lymphoma tumor masses on both the malignant cells and the supportive TME (35, 36). Recent studies show that CD123 is also expressed in a subset of myeloid-derived suppressor cells (MDSC; refs. 37). The objective of this study was therefore to develop an anti–Hodgkin lymphoma CART immunotherapy that would also be able to overcome the immunosuppression of the Hodgkin lymphoma microenvironment by targeting the TME. We confirmed the presence of CD123 on HRS cells and found that many immune cells in the Hodgkin lymphoma TME, in particular immunosuppressive M2-type TAMs, express CD123. We previously developed anti-CD123 CART T cells for the treatment of acute myeloid leukemia (AML; ref. 38), and we now find that CART123 cells can eradicate disseminated Hodgkin lymphoma tumor xenografts, leading to durable remissions and the development of immune memory. Furthermore, through cotargeting of immunosuppressive CD123-expressing TAMs, CART123 cells are shown to be resistant to microenvironmental immunosuppression.

RESULTS

The IL3 Receptor α, CD123, Is Expressed in Hodgkin Lymphoma Cells and in TAMs

We sought to identify a tumor-associated antigen expressed in HRS cells and in cells of the Hodgkin lymphoma TME. For this purpose, we analyzed RNA expression on microdissected HRS cells from 29 Hodgkin lymphoma biopsies (dataset GSE39133; ref. 39), and we found high expression of the IL3 receptor α, CD123, in the vast majority of patients compared with control B cells (Fig. 1A), similar to what was observed for CD30 (Supplementary Fig. S1A). To confirm this finding and to study the expression of CD123 in the TME, we evaluated the expression of CD123 and CD30 on histologic specimens from patients with Hodgkin lymphoma (Fig. 1B). As expected, in 10 of 10 of the Hodgkin lymphoma tissue biopsies, HRS cells were positive for CD30, the hallmark biomarker of HRS. Importantly, in 5 of 10 specimens, we also detected expression of CD123 on the HRS cells by IHC. IHC revealed that CD30 was sparsely distributed on infiltrating immune cells, whereas CD123 was highly expressed on the TME (Fig. 1B); in particular, we found that TAMs expressed CD123, as shown by dual-color immunofluorescence and IHC (Fig. 1C). We confirmed the expression of CD123 in the Hodgkin lymphoma TME by analyzing RNA expression data of whole biopsies from 130 patients with Hodgkin lymphoma (dataset GSE17920; ref. 20) and found a correlation between CD123 expression with the two standard macrophage markers, CD68 and CD11b (Fig. 1D). To define an appropriate human tumor model for further studies, we evaluated four Hodgkin lymphoma cell lines (HDLM-2, KM-H2, SUP-HD1, and L-428) and found CD123 to be highly expressed by these cells (CD30 used as positive control) at both the mRNA (GSE39133; ref. 39; Supplementary Fig. S1B) and protein level (Fig. 1E and Supplementary Fig. S1C, respectively).

Hodgkin Lymphoma Cells Polarize Normal Macrophages to an M2-Like Phenotype

Because TAMs are involved in Hodgkin lymphoma pathogenesis and affect its prognosis (20, 25), we sought to examine whether Hodgkin lymphoma cells can directly mediate conversion of macrophages to an immunosuppressive phenotype. Human normal donor macrophages differentiated from peripheral blood monocytes (see macrophage differentiation protocol in Supplementary Fig. S2A) were cocultured with HDLM-2 cells, IL4 (a standard M2-polarizing cytokine), or a control non–Hodgkin lymphoma cell line (the B-cell acute lymphoblastic leukemia cell line NALM-6). After 24 hours of coculture, macrophage phenotype was analyzed by flow cytometry using a panel of 6 markers (CD206, CD163, CD86, CD80, PD-L1, and PD-L2). HDLM-2–primed macrophages showed an M2-like phenotype, with expression of CD206 and CD163 similar to that of IL4-primed macrophages (Fig. 2A; refs. 40–44). In particular, PD-L1 was significantly increased in macrophages exposed to Hodgkin lymphoma cells (Supplementary Fig. S2B and S2C). As a control, macrophages cocultured with NALM-6 showed a non–M2-like phenotype (M0). Notably, exposure of macrophages to HDLM-2 supernatants led to phosphorylation of STAT6, as observed during IL4 activation (Fig. 2B). Importantly, CD123 expression was maintained in both IL4 and Hodgkin lymphoma–polarized macrophages (Fig. 2C).

In order to test the function of the phenotypically defined immunosuppressive macrophages, we cocultured human CART cells with macrophages under M0, M2 (IL4 induced), or Hodgkin lymphoma–polarized macrophages. In this experiment, we used the anti-CD19 CART as the reference “responder” cells and the CD19+ acute leukemia B-cell line NALM-6 as the “stimulator” cells (45). As expected, CART19 strongly proliferated in the presence of the target cell line, but this proliferation was slightly reduced in the presence of M2 macrophages and further inhibited by Hodgkin lymphoma TAMs (Fig. 2D and E). In order to probe the mechanism of macrophage polarization by Hodgkin lymphoma cells, we analyzed the concentration of cytokines and chemokines in the supernatant of four Hodgkin lymphoma cell lines and two control non-lymphoma cell lines (NALM-6 and K562) using the Luminex assay (Fig. 2F). Hodgkin lymphoma cells secreted cytokines and chemokines capable of stimulating and recruiting myeloid cells, such as GM-CSF, CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10, as well as the M2 polarizing cytokines IL13, IL6, and IL10. The expression of M2 polarizing cytokines (IL13, IL10, and IL6), macrophage supporting factors [GM-CSF (encoded by CSF1) and M-CSF (encoded by CSF1)], and the immunosuppressive checkpoint protein PD-L1 (encoded by CD274) were confirmed by RNA expression analysis of microdissected HRS cells (n = 29, dataset GSE39133; Fig. 2G).

Anti-CD123 Chimeric Antigen Receptor T Cells Kill Hodgkin Lymphoma Cells In Vitro and In Vivo

Having demonstrated the presence of CD123 in the HRS cells, we sought to demonstrate the extent to which anti-CD123 CART cells (CART123; see CAR123 construct in Supplementary Fig. S3A) can recognize Hodgkin lymphoma cells, as measured...
by antigen-dependent CART proliferation, cytokine production, and specific tumor lysis. We used Hodgkin lymphoma cell lines as models, given that it is impossible to propagate primary Hodgkin lymphoma in culture and due to the lack of reliable primary Hodgkin lymphoma xenograft models. In vitro, CAR123+ T cells but not CAR123− T cells demonstrated specific CD107a degranulation and intracellular expression of cytokines (IFNγ, IL2, and TNFα) when cocultured with Hodgkin lymphoma cells for 4 to 6 hours (Fig. 3A). After 24 hours, potent cytotoxicity against HDLM-2 was exerted by CART123, but not by the control untransduced T cells (UTD; Fig. 3B). Complete eradication of Hodgkin lymphoma cells by day 4 was associated with massive CART123 expansion (day 20) (Fig. 3C). Notably, additional Hodgkin lymphoma cell lines (L-428, KM-H2, and SUP-HD1) were also efficiently killed in vitro by CART123 (Supplementary Fig. S3B). CART123 proliferated when cocultured with Hodgkin lymphoma cells (HDLM-2 or KM-H2), as demonstrated by absolute T-cell number after 5 days (Fig. 3D) and carboxyfluorescein succinimidyl ester (CFSE) dilution (Fig. 3E). Importantly, CART123 cells secreted effector cytokines such as GM-CSF, IFNγ, MIP1β, and TNFα in the presence of Hodgkin lymphoma cells (Fig. 3F).

We then developed a xenograft model of progressive Hodgkin lymphoma by injecting $1 \times 10^6$ luciferase+ HDLM-2 cells intravenously in NSG mice on day 0 (Fig. 4A). Serial bioluminescent imaging (BLI) demonstrated tumor engraftment by day 7, which was followed by gradual increase in tumor burden over approximately 6 weeks. At day 42, when the tumor burden...
was 20-fold higher than the baseline, mice were treated with 1.5 × 10^6 CART123 cells or control T cells. CART123 induced complete and durable eradication of disseminated tumor within 14 days, leading to 100% relapse-free and 100% overall survival at 6 months (Fig. 4B and C). Mice were followed up for almost 1 year, and no relapses were observed in CART123-treated mice, whereas mice treated with control T cells had a median survival of 128 days (P = 0.009). Minimal reduction in tumor progression was observed in UTD mice; however, the overall survival of UTD versus no treatment was not statistically different. Tumor elimination was associated with extensive CART expansion in the peripheral blood, including both CD8+ and CD4+ cells as detected by flow cytometry in serial peripheral blood analyses, as seen in clinical studies of anti-CD19 CART cells (Fig. 4D and E). Importantly, the in vivo efficacy of CART123 against Hodgkin lymphoma was confirmed in a second xenograft model where NSG mice were injected with luciferase-positive SUP-HD1 cells and treated with CART123 at day 21. CART123-treated mice reached complete remission whereas controls progressed (Supplementary Fig. S3C).

**CART123 Cells Establish Long-Term Immunologic Memory in Mice with Hodgkin Lymphoma**

Long-term persistence and T-cell memory play an important role in immunosurveillance and prevention of relapse. In order to demonstrate the formation of immunologic memory, at a late time point (day 250) when CART123-treated mice were in remission, we rechallenged them with HDLM-2...
Figure 3. Anti-CD123 chimeric antigen receptor T cells exert potent effector function against Hodgkin lymphoma in vitro. A, Hodgkin lymphoma cells (HDLM-2) were cocultured with CART123 for 4 to 6 hours. CAR+ but not CAR T cells expressed high levels of the degranulation marker CD107α and produced intracellular cytokines like IFNγ, IL2, and TNFα. B, CART123, but not UTD, exert potent cytotoxicity (luciferase-based killing assay) against Hodgkin lymphoma cells in a dose-dependent manner. C, Hodgkin lymphoma cells (HDLM-2) were cocultured at long term with CART123 or control UTD. At day 20, CART123 but not UTD killed Hodgkin lymphoma cells and proliferated. D, CART123 or UTD were cocultured with media, PMA/ionomycin (P-I; positive control) or two Hodgkin lymphoma cell lines (HDLM-2 and KM-H2) for 5 days; CART123 but not UTD controls showed significant proliferation as shown by absolute number and CFSE dilution. E, Hodgkin lymphoma cells stimulated CART123 but not UTD cells to release multiple cytokines including GM-CSF, IFNγ, MIP1β, and TNFα. E.T. effector–target ratio. **, P < 0.01; ns, not significant (P > 0.05).
Figure 4. Anti-CD123 chimeric antigen receptor T cells exert potent effector function against Hodgkin lymphoma in vivo. A, Experiment schema: $2 \times 10^6$ luciferase-positive HDLM-2 cells were injected i.v. in NSG mice and tumor engraftment was monitored by bioluminescence imaging (BLI). At day 42 mice were randomized to receive no treatment, $2 \times 10^6$ control untransduced T cells (UTD), or $2 \times 10^6$ CART123. B, Mice receiving CART123, but not controls, experienced complete response with long-term remission of disease (>250 days), representative experiment. C, CART123-treated mice have a significantly longer overall survival as compared with controls (3 experiments combined). D, CART123 cells engraft, expand, and disappear from the peripheral blood after clearing the tumor. E, T cells in the peripheral blood of CART123-treated mice were both CD8 and CD4 with high expression of the CAR.
Hodgkin lymphoma cells (see experiment schema in Fig. 5A). As a control, we used tumor- and T cell–naïve mice. Notably, in the mice previously treated with CART123 the tumor was rejected and there was no sign of long-term relapse; in contrast, in control mice, Hodgkin lymphoma engrafted and progressed over time (Fig. 5B). The rejection was associated with a reexpansion of the previously undetectable CART123 cells in the peripheral blood (∼10 months after T-cell injection; Fig. 5C). In contrast, in control mice, Hodgkin lymphoma cells engrafted and led to the death of these mice (Fig. 5D).

CART123 Cells Are Resistant to Inhibition by M2 Macrophages

Lastly, as we have demonstrated that CART cells can be inhibited by M2- and Hodgkin lymphoma–polarized macrophages and that these macrophages are CD123-positive, we sought to determine if CART123 cells were also susceptible to macrophage inhibition, or conversely, due to the expression of CD123 in Hodgkin lymphoma macrophages, they would receive additional stimulation.

We generated immunosuppressive M2 macrophages by culturing them with HDLM-2 cells (see Supplementary Fig. S2A). Using the preestablished CART19 and B-cell acute lymphoblastic leukemia model, we showed that Hodgkin lymphoma–polarized TAMs can reduce CART19 proliferation following CAR stimulation at day 5 but, in stark contrast, CART123 cells were not affected (Fig. 6A). Importantly, CART123 cells actively recognized M2 (both IL4 polarized or Hodgkin lymphoma TAMs) macrophages, formed aggregates around them as shown by microscopy at 24 hours (Fig. 6B, left), and exerted significant cytotoxicity by day 5 as shown by flow cytometry (Fig. 6B, right). Moreover, cytokine production by T cells was inhibited by M2 macrophages in CART19 but not in CART123 (Fig. 6C). We demonstrated that the ability of CART123 cells to overcome immunosuppression relies on their capacity to recognize and efficiently kill macrophages, as shown in Supplementary Fig. S4A and S4B. CART123 cells thereby overcome TAM-mediated inhibition by simultaneous targeting of CD123 on HRS cells and macrophages.

DISCUSSION

Hodgkin lymphoma is usually associated with a good prognosis, but a subset of patients experience multiple relapses...
or is primarily refractory. Recent advances in the care of Hodgkin lymphoma, including the antibody–drug conjugate BV or the immune checkpoint antagonists nivolumab and pembrolizumab, confer high overall response rates in this patient population, but the rate of complete responses is relatively low (3). CART cell therapy has recently come to the fore as a potent antigen-specific modality in the treatment of B-cell malignancies (46). Because CD19 is not expressed on the malignant HRS cells, CART cell therapy against the commonly expressed CD30 antigen has been attempted, but the results have been disappointing to date (18). One possibility is that the heavy microenvironmental infiltrate in Hodgkin lymphoma, composed of numerous immunosuppressive cells such as TAMs, inhibits the activity of CART cells. Indeed, CD68+ macrophage infiltration correlates with a poor prognosis in Hodgkin lymphoma (20). To our knowledge, none of the currently approved therapies against Hodgkin lymphoma specifically target the TME, although it is distinctly possible that the PD-1 antagonists block the interaction between PD-1 on T cells and PD-L1 on HRS cells as well as on TAMs. In this context, we sought an antigen that is coexpressed on HRS cells and in the TME, and hypothesized that a cotargeting approach would circumvent immunosuppressive stimuli from the TME.

Using several orthogonal techniques, including analysis of publicly available gene expression profiling of microdissected primary HRS cells and primary Hodgkin lymphoma lesions, IHC, and flow cytometry, we found and confirmed previous reports that CD123 is expressed on the majority of HRS cells and in the TME (29, 33). We have previously described the activity of CART123 in human AML (38). Here, we show that CART123 cells specifically degranulate, proliferate, produce cytokines, and kill target Hodgkin lymphoma cells in vitro. In vivo, we show that human CD123-redirected T cells display potent therapeutic activity against disseminated Hodgkin lymphoma, persist long-term after eradication of disease, and are capable of mounting a robust recall response to tumor challenge. Because the xenograft system does not permit an evaluation of the role of the TME, we turned to an in vitro

**Figure 6.** CART123 cells are resistant to inhibition by M2 macrophages. A, In a 5-day CFSE proliferation assay, CART123 cells are completely resistant to Hodgkin lymphoma-polarized macrophage-mediated inhibition. B, CART123 cells rapidly (day 1) recognized M2 macrophages, forming clusters around macrophages and clearing them by day 5 as shown by phase-contrast microscopy (20X) and flow cytometry, respectively. C, CART19 but not CART123 cytokine secretion was significantly reduced in the presence of M2 (IL4-polarized) macrophages in vitro. *, P < 0.05; **, P < 0.01.
system to investigate the role of the TME in resistance to CART cells.

We showed that Hodgkin lymphoma cell lines produce several macrophage-attracting chemokines (CCL2-5 and CXCL9-10) and immunosuppressive cytokines, including IL13, IL10, and IL6, and we demonstrated activation of the STAT6 signaling pathway in response to Hodgkin lymphoma-conditioned media, which is a known IL4–IL13 signaling pathway. IL13 has previously been reported to play a role in autocrine growth stimulation of HRS cells (47, 48) and has been postulated to mediate recruitment of immune cells into Hodgkin lymphoma TME. We showed that exposure to Hodgkin lymphoma supernatant polarizes macrophages toward the “M2” phenotype, leading to a consequent immunosuppressive effect on CART cells. We used anti-CD19 CART cells (CART19) stimulated by the B-cell leukemic cell line NALM-6 as a “gold standard” for potent antigen-specific stimulation (45, 49). We found that Hodgkin lymphoma–exposed macrophages, as a model of TAMs, are able to suppress CART19 stimulation by CD19. Although CD19 is not expressed on Hodgkin lymphoma and CART19 therapy is not expected to have activity in Hodgkin lymphoma, there is evidence that clonotypic CD19+ B cells in Hodgkin lymphoma and leading to B-cell–directed therapies in Hodgkin lymphoma (50, 51), including a trial at our institution evaluating CART19 in this malignancy (NCT02277522). More importantly, if our findings are generalizable to TAMs in other malignancies, TAMs may have a similar effect on CART19 in *bona fide* CART cells.

CD123 is expressed in other hematologic neoplasms, including AML, plasmacytoid dendritic cell neoplasm (52), hairy cell leukemia (53), and B-cell acute lymphoblastic leukemia (54–57). Due to these characteristics, multiple modalities to target CD123 in hematologic neoplasms (including Hodgkin lymphoma; refs. 58 and 59) have been developed, in particular the IL3–diphtheria toxin fusion protein (SL-401, SL-501, and DT388IL3; refs. 60–62), unconjugated anti-CD123 monoclonal antibodies (CSL-360 and CSL-362; ref. 63), antibody–drug conjugates (64), bispecific antibodies (65, 66), or CD3Fv–IL3 fusion constructs (67), and, more recently, anti-CD123 chimeric antigen receptor T cells (30, 38, 68, 69). Notably, currently 13 clinical trials are investigating CD123 as a target for hematologic cancers (clinicaltrials.gov). Our previous publication on the antileukemia efficacy of anti-CD123 CAR T cells also highlighted the potential for myeloablation resulting from targeting CD123 on normal hematopoietic precursors (38). Thus, our current clinical trial utilizes short-acting mRNA-electroporated CART rather than permanently modified lentivirally transduced CART cells (NCT02623582). A recent case report described one patient treated with lentivirally transduced CART123 showing the feasibility of this approach, supported by preliminary results from other CD123-targeted agents (63, 70, 71). If the predicted CART123 myelotoxicity is confirmed in ongoing trials, we propose that CART123 could be depleted once remission is obtained (using clinically available anti–T-cell antibodies; ref. 72) prior to a subsequent SCT. Because SCT is already a standard strategy for r/r Hodgkin lymphoma, and metabolic remission prior to SCT is an important prognostic factor, it may be possible to use adoptive cellular therapy with potent CART123 to achieve a complete remission prior to salvage SCT.

In summary, we found that Hodgkin lymphoma cells can polarize macrophages to an immunosuppressive phenotype. These “M2”–like TAMs are present within the TME, express CD123, and are targetable by CD123-directed CART cells. CART123 cells display potent therapeutic activity against disseminated Hodgkin lymphoma in two xenograft models. Thus, this approach could be used for depleting both malignant cells and the surrounding immunosuppressive milieu.

**METHODS**

**Cell Lines and Primary Samples**

Cell lines were originally obtained from ATCC (K-562) or DSMZ (HDLM-2, KM-H2, L-428, SUP-HD1, MOLM-14, and NALM-6). Cell line authentication was based on extensive flow cytometry characterization (data not shown). Cell lines were obtained between 2009 and 2013 and were used for experiments within 15 to 20 passages. All cell lines were tested for the presence of *Mycoplasma* contamination (MycopAlert Mycoplasma Detection Kit, LT07-318, Lonza). For some experiments, cell lines were transduced with luciferase (click-beetle green) and/or eGFP and then sorted to obtain at least 99% positive population. MOLM-14 and K-562 were used as controls, as indicated in the relevant figures. The cell lines were maintained in culture with RPMI-1640 (Gibco, 11875-085, Life Technologies) supplemented with 10% FBS (Gibco, 100-106), and 50 UI/mL penicillin/streptomycin (Gibco, Life Technologies, 15070-063). For all functional studies, primary cells were chased for at least 12 hours before experiment and rested at 37°C. Deidentified formalin-fixed primary human Hodgkin lymphoma specimens were obtained from the clinical practices of the University of Pennsylvania/Children’s Hospital of Philadelphia under an Institutional Review Board protocol.

**IHC**

IHC of formalin-fixed paraffin-embedded tissue was performed using antibodies against human CD30 (DAKO M0751), CD68 (Leica PA0273, clone 514H12) RTU and CD123 (Leica NCL-L-CD123) at 1:150 dilution. For the dual IHC, staining was done sequentially on a Leica Bond-IIITM instrument using the Bond Polymer Refine Detection System (Leica Microsystems DS9800-DAB for CD68 and DS9390-RED for CD123). Heat-induced epitope retrieval was done for 20 minutes with ER2 solution (Leica Microsystems AR9640). Incubation time with the CD68 antibody was 15 minutes followed by 8-minute post-primary step and 8-minute incubation with polymer horseradish peroxidase (HRP), then block endogenous peroxidase for 5 minutes, followed by 10 minutes of DAB. After the first antibody staining was completed, the slides were incubated with the anti-CD123 antibody for 15 minutes, followed by post-primary alkaline phosphatase for 20 minutes and post-polymer for 20 minutes. Subsequently, the slides were stained with Fast red for 7 minutes. Slides were washed three times between each step with bond wash buffer or water. All the experiments were done at room temperature.

**Immunofluorescence**

Immunofluorescence of formalin-fixed paraffin-embedded tissue was performed using antibodies against human CD23 (Leica NCL-L-CD23) at 1:400 plus C68 (Dako M0814) at 1:16K double staining sequentially. Staining was done manually by using Pekin Elmer Opal 7 TIL Panel B Kit (Opal 7B). Heat-induced epitope retrieval was done for 15 minutes with AR9 buffer. Incubation time with the primary antibody was 30 minutes followed by 30-minute incubation with polymer HRP, then add on Opal 520 (FITC) for 10 minutes for CD123, and Opal 570 (Texas Red) for CD68. All the experiments were done at room temperature. Slides were washed three times between each step with 1xTBS buffer or water.
Gene Expression Analysis

To interrogate gene expression in primary Hodgkin lymphoma and in multiple Hodgkin lymphoma–derived cell lines, the BioGPS web tool was used (http://biogps.org/). For primary Hodgkin lymphoma, gene expression values of microdissected HRS cells (n = 29 patients) were compared with normal microdissected germinal center B cells (n = 5 patients) from Affymetrix GeneChip data (dataset GSE-39133; ref. 39). For Hodgkin lymphoma–derived cell line gene expression assessment, five Hodgkin lymphoma cell lines (KM-H2, L-428, L-540, L-1236, and HDLM-2) were compared with microdissected germinal center B cells from five donors (dataset GSE-39132; ref. 39). Expression data of diagnostic non-microdissected biopsies from 130 patients with Hodgkin lymphoma (dataset GSE17920; ref. 20) were used to correlate expression of CD123 to macrophage markers CD68 and CD11b. Statistical significance was determined by the Student t test.

Multiparametric Flow Cytometry

Flow cytometry was performed as previously described (73). Anti-human antibodies were purchased from BioLegend, eBioscience, or Becton Dickinson. For cell number quantitation, Countbright (Invitrogen) beads were used according to the manufacturer’s instructions. In all analyses, the population of interest was gated based on forward versus side scatter characteristics followed by single gating and, live cells were gated using Live Dead Fixable Aqua (Invitrogen). Time gating was included for quality control. Detection of CAR123 was performed using goat–anti-mouse antibody (Jackson Laboratories) or CD123-Fc/His (Sino Biologicals) and anti-His-APC (R&D) or PE (AbCam) or directly PE-conjugated CD123 protein (Sino Biologicals and AbCam). Flow cytometry was performed on a four-laser Fortessa-LSR II cytometer (Becton-Dickinson) and analyzed with FlowJo X 10.0.7r2 (TreeStar).

Human Macrophage Differentiation

As shown in Supplementary Fig. S1A, human macrophages (M0) were generated by differentiating positively selected CD14+ normal donor monocytes (Human CD14 MicroBeads, Miltenyi Biotech) for 7 days in RPMI-1640 (Gibco) supplemented with 10% FBS (Gemini BioProducts), 1% Glutamax (Gibco), 10 ng/mL recombinant human GM-CSF (Peprotech), and 1% penicillin/streptomycin (Lonza). Macrophages were polarized to M2 by adding either 20 ng/mL human IL4 or IL13 (Peprotech) to the differentiation media for an additional 24 hours. Hodgkin lymphoma TAMs were generated by culturing macrophages with HDLM-2 cells or adding HDLM-2-conditioned media for 24 hours after differentiation.

Macrophage Killing Assay

Primary human monocyte–derived macrophages were differentiated and polarized as described above. Autologous macrophages were cocultured with UTD T cells, CART123, or media alone for 7 days at an effector–target ratio (E/T) of 1:1 in a 96-well plate. All conditions were tested in triplicate. At the end of a 7-day coculture period, killing was assessed by 10x phase microscopy and FACS analysis. Live macrophages were identified as CD11b+LiveDeadAqua. Absolute macrophage counts were determined using CountBright Absolute Counting Beads (Thermo Fisher), and statistical analysis was done using the Student t test.

Generation of CAR Constructs and CART Cells

The second-generation anti-CD123 chimeric antigen receptor (CAR123) features an anti-CD123 scFv (clone 32716), CD8 hinge, 4-1BB costimulatory domain, and CD3-ζ signaling domain (Supplementary Fig. S3A; ref. 38). This construct is currently used in a clinical trial for AML at the University of Pennsylvania (NCT02623382). The murine anti-CD19 chimeric–antigen receptor (CD8 hinge, 4-1BB costimulatory domain, and CD3 zeta signaling domain) was generated as previously described (45, 74). This is the same construct currently used in the CART19 (CTL019) clinical trials at the University of Pennsylvania. Production of CAR-expressing T cells was performed as previously described (38). Normal donor CD4 and CD8 T cells or peripheral blood mononuclear cells (PBMC) were obtained from the Human Immunology Core of the University of Pennsylvania. Prior to all experiments, T cells were thawed and rested overnight at 37°C.

In Vitro T-cell Effector Function Assays

Degranulation, CFSE proliferation, cytotoxicity assays, and cytokine measurements were performed as previously described (10, 38, 75). Phase-contrast images of human macrophage and T-cell coculture (at 24 hours) were generated using the 20x lens on a Nikon Eclipse Ti-S microscope (Nikon Instruments, Inc.)

Animal Experiments

In vivo experiments were performed as previously described (73). Schemata of the utilized xenograft models are discussed in detail in the relevant figures. NOD/SCID gamma chain deficient (NSG) mice originally obtained from Jackson Laboratories were purchased from the Stem Cell and Xenograft Core of the University of Pennsylvania. Cells (Hodgkin lymphoma cell lines or T cells) were injected in 100 to 200 μL of PBS at the indicated concentration into the tail veins of mice. Bioluminescent imaging was performed using a Xenogen IVIS-200 Spectrum camera and analyzed with LivingImage software v. 4.3.1 (Caliper LifeSciences). Animals were euthanized at the end of the experiment or when they met prespecified endpoints according to the Institutional Animal Care and Use Committee (IACUC) protocols.

Study Approval

Animal experiments were performed according a protocol (#803230) approved by the IACUC that adheres to the NIH Guide for the Care and Use of Laboratory Animals.

Statistical Analysis

All statistics were performed as indicated using GraphPad Prism 6 for Windows, version 6.05. Two-tailed Student t test was used to compare two groups; in analysis where multiple groups were compared, one-way analysis of variance (ANOVA) was performed with Holm–Sidak correction for multiple comparisons. Linear regression was used to correlate CD123 RNA expression with CD11b or CD68. When multiple groups at multiple time points/ratios were compared, Student t test or ANOVA for each time point/ratio was used. Survival curves were compared using the log-rank test. In the figures, asterisks are used to represent P values (*, <0.05; **, <0.01; *** <0.001; ****, <0.0001), and “ns” means “not significant” (P > 0.05).

Disclosure of Potential Conflicts of Interest

M. Ruella reports receiving research support from Novartis as an inventor in patents. S.S. Kenderian has ownership interest (including patents) in Novartis. C.H. June reports receiving a commercial research grant from Novartis and has ownership interest in an IP licensed to Novartis by the University of Pennsylvania. S. Gill reports receiving research funding from Novartis Pharmaceuticals and has ownership interest in patents. No potential conflicts of interest were disclosed by the other authors.

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