CD74: A New Candidate Target for the Immunotherapy of B-Cell Neoplasms

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Abstract CD74 is an integral membrane protein that functions as a MHC class II chaperone. Moreover, it has recently been shown to have a role as an accessory-signaling molecule and has been implicated in malignant B-cell proliferation and survival. These biological functions combined with expression of CD74 on malignant B cells and limited expression on normal tissues implicate CD74 as a potential therapeutic target. The anti-CD74 monoclonal antibody LL1 has been humanized (hLL1 milatuzumab or IMMU-115) and can provide the basis for novel therapeutic approaches to B-cell malignancies, particularly because this antibody shows rapid internalization into $CD74^+$ malignant cells. This article reviews the preclinical evaluations of LL1, its humanized form, and isotope, drug, and toxin conjugates. These studies show that unconjugated hLL1 and conjugates of hLL1 constructs with radioisotopes, doxorubicin, and frog RNase have high antitumor activity in non – Hodgkin's lymphoma and multiple myeloma *in vitro* and in tumor xenograft models. Single-dose studies of hLL1 in monkeys showed no adverse effects but did decrease circulating B and T lymphocytes and natural killer cells. When evaluated in combination with rituximab, either equivalent or improved efficacy, compared with either antibody alone, was observed. CD74 is a new candidate target for the immunotherapy of neoplasms expressing this antigen, which can be exploited using either a naked antibody or conjugated to isotopes, drugs, or toxins.

CD74 (invariant chain, Ii) is a type II transmembrane glycoprotein that associates with the MHC class II α and β chains and directs the transport of the αβIi (invariant chain) complexes to endosomes and lysosomes (1–4). In addition, CD74 is involved in signaling pathway functioning as a survival receptor (5). These biological functions, combined with expression of CD74 on malignant B cells and limited expression on normal tissues, implicate CD74 as a potential therapeutic target. The anti-CD74 monoclonal antibody (mAb) LL1 has been humanized (hLL1 milatuzumab or IMMU-115) and can provide the basis for novel therapeutic approaches to B-cell malignancies. This article reviews the preclinical evaluations of LL1, its humanized form, and isotope, drug, and toxin conjugates. The evidence suggests that hLL1 is a promising candidate antibody for the therapy of CD74-expressing malignancies, such as non – Hodgkin's lymphoma (NHL) and multiple myeloma (MM). Due to its rapid internalization property, hLL1 also shows considerable promise as a drug, isotope, or toxin immunoconjugate.

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CD74 Molecule: Structure, Function, and Expression

CD74 has 30 NH_2-terminal, intracytoplasmic amino acid residues, a 26-amino acid hydrophobic transmembrane region, and a 160-amino acid extracytoplasmic domain containing two N-linked carbohydrate chains (1, 6). Once synthesized, CD74, DRα, and DRβ begin to associate within the endoplasmic reticulum. This is believed to occur by sequential addition of DRα/β heterodimers to a trimeric core of CD74 molecules until a nine-subunit complex with equimolar amounts of these three chains is formed (7, 8). DR-CD74 complexes then progressively traffic to the late endosomal compartment where CD74 is cleaved into peptide fragments by proteases. These CD74 cleavage fragments have reduced affinity for DR and allow for the displacement of CD74 by peptides present in the endosome. The DR-peptide complexes then traffic to the cell surface for antigen presentation. During this process, a sizable DR-CD74 pool transiently resides on the cell surface.

In addition to its role as a chaperone molecule for MHC class II, CD74 has been shown to function as a signaling molecule in several pathways. CD74 was shown to be directly involved in the maturation of B cells through a pathway involving nuclear factor-κB (9, 10). Using activating anti-CD74 mAbs, Starlets et al. (5) showed recently that syk, phosphatidylinositol 3-kinase, and AKT are activated following ligation of CD74 on murine B cells and that activation of nuclear factor-κB occurs with downstream transcription of antiapoptotic genes, such as *Bcl-X_L*. Recently, *Helicobacter pylori* was shown to bind CD74 on gastric epithelial cells and stimulate nuclear factor-κB and interleukin-8 production, thus corroborating the involvement

Table 1. LL1 immunohistology of patient biopsy specimens

Diagnosis	No. positive/ no. tested	% Target cells stained
Follicular lymphoma	8/9	>95%
Diffuse large B-cell lymphoma	4/4	~80%
Other NHL	31/35	ND
Chronic lymphocytic leukemia/ small lymphocytic lymphoma	14/14	>90%
MM	19/22	16/22, >95%; 3/22, ~50%
Waldenström's macroglobulinemia	2/2	30-50%

Abbreviation: ND, not determined.

of nuclear factor-κB and implicating interleukin-8 in the signal cascade initiated by CD74 activation (11, 12).

CD74 has also been shown to be the high-affinity receptor for the proinflammatory cytokine macrophage migration inhibitory factor (13). Macrophage migration inhibitory factor is ubiquitously expressed and contributes to innate immune system activation in macrophages and monocytes through activation of the extracellular signal-regulated kinase signaling pathway (13, 14). Numerous reports have shown that macrophage migration inhibitory factor inhibits p53 function (15, 16), activates components of the mitogen-activated protein kinase and Jun activation domain-binding protein-1 pathways (14, 17, 18), antagonizes the action of glucocorticoids (19), and up-regulates Toll-like receptor 4 (20). Macrophage migration inhibitory factor is overexpressed in solid tumors (21, 22) and related to tumor progression (23–25). Recently, CD44 has been reported to be an integral member of the CD74 receptor complex leading to macrophage migration inhibitory factor signal transduction (26), substantiating earlier reports of a functional and cell surface association between CD74 and CD44 (27).

In normal tissues, CD74 is expressed on HLA class II–positive cells, including B cells, monocytes, macrophages, Langerhans cells, dendritic cells, subsets of activated T cells, and thymic epithelium. Under inflammatory conditions, CD74 expression may be observed on endothelial and certain epithelial cells (28). CD74 is also expressed on a variety of malignant cells. Its expression has been observed in ~90% of B-cell cancers evaluated as well as the majority of cell lines derived from these cancers (29, 30). Table 1 summarizes immunohistochemical staining of patient biopsy specimens with the anti-CD74 mAb LL1. Staining of a trephine bone biopsy from a MM patient with the anti-CD74 mAb LL1 is shown in Fig. 1. CD74 staining is seen in >95% of MM plasma cells. Malignant plasma cells from adjacent histologic sections were identified with an anti-CD138 mAb. This specimen was among a group of 22 such biopsy specimens tested for CD74 expression. Nineteen of these 22 specimens exhibited positive staining (i.e., >50% of MM cells), with 16 showing >95% of the MM cells strongly reactive, as exemplified in Fig. 1A. This group of cases was also assessed for HLA-DR expression. As seen in Fig. 1B, considerably less staining was seen with the anti-HLA-DR mAb (TAL.1B5) in the MM cases. In fact, only 1 of the 19 $CD74^+$ cases showed >50% HLA-DR positivity. This is in contrast with most normal and malignant cell types, which coexpress DR and CD74. This finding may have implications for the antigenicity of MM cells and their ability to evade recognition by the host immune system.

CD74 expression has also been found in nonhematologic malignancies, including gastric (31), renal (32), non–small cell lung (33), and thymic epithelium neoplasms (34), certain types of sarcoma (fibrous histiocytoma; ref. 35), and atypical fibroxanthoma, an unusual malignant fibrohistiocytic tumor of sun-damaged skin (36). CD74 expression in many of these cancers has been suggested to be a prognostic factor, with higher relative rates of CD74 behaving as a marker of tumor progression or poor clinical outcome (37). This correlation may be related to suppressive effects on host immune responses.

Anti-CD74 mAb LL1

The murine LL1 mAb (IgG1κ) was generated by hybridoma technology after immunization of BALB/c mice with Raji human Burkitt lymphoma cells (38). The CD74 specificity of LL1 was first suggested by immunofluorescence and immunohistology studies that showed discrimination between lymphoid and nonlymphoid tissues, reactivity with malignant B-cell tissues sections and cloned B-cell lymphomas, and molecular size determinations by Western immunoblots of cell extracts. Confirmation that LL1 binds to a cell surface–expressed epitope

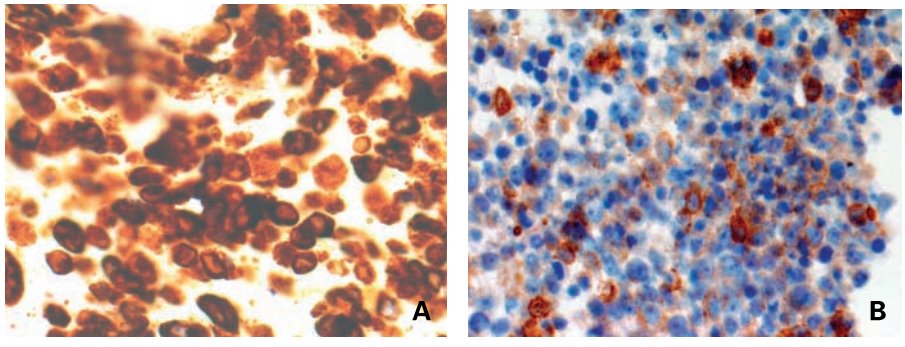

Fig. 1. Immunohistochemical staining of trephine bone marrow biopsy of a MM patient. *A,* stained with mAb LL1 (anti-CD74). *B,* stained with mAb TAL.1B5 (anti-HLA-DR α chain). Immunohistochemistry was done by indirect immunoperoxidase staining of tissue sections with hematoxylin counterstain as described previously (29).

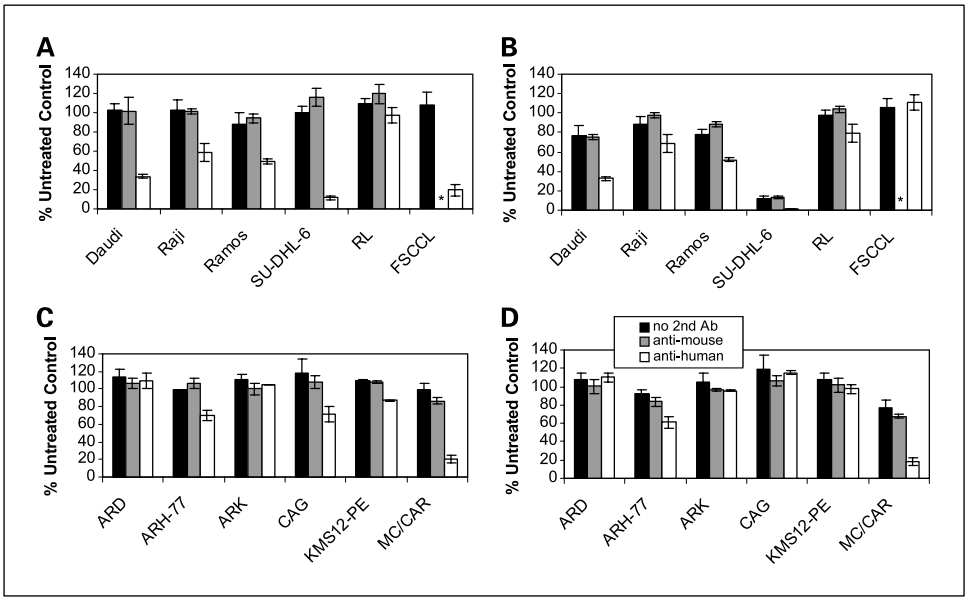

Fig. 2. hLL1 inhibits proliferation of cell lines. Antiproliferative effects of the anti-B-cell mAbs were assessed by measuring the uptake of [^{3}H]thymidine. Cells were cultured with the mAbs with or without a second antibody for cross-linking to mimic the role of effector cells or cross-linking molecules present *in vivo*. Black columns, no second antibody; gray columns, goat anti-mouse IgG; white columns, goat anti-human IgG. Bars, SD. *A,* NHL cell lines incubated with hLL1. *B,* NHL cell lines incubated with rituximab. *C,* MM cell lines incubated with hLL1. *D,* MM cell lines incubated with rituximab. Asterisk, not determined. Reproduced with permission from Stein et al. (30).

of CD74 was then provided by competitive binding experiments and immunoprecipitation studies in conjunction with other anti-CD74 antibodies. Binding was inhibited by mAb LN2, which reacts with a cell surface epitope, but not BU45 or POP.I4.3, which does not. LL1 is unique in that although it reacts with the LN2 epitope its cell surface binding is markedly stronger than that of LN2 (39).

One of the most interesting properties of LL1 was uncovered by following the fate of the antibody after binding to the cell surface. B-cell lymphomas were found to take up and catabolize $\sim 10^7$ LL1 molecules per day, a much faster rate than that of other antibodies that are considered to be rapidly internalized, such as CD19, CD22, and anti–transferrin receptor antibodies (39). Moreover, the internalization of LL1 differs from that of the anti–growth factor receptors, another class of mAbs showing rapid internalization (including anti–epidermal growth factor receptor), because it is not dependent on antibody cross-linking. An additional notable difference is that, with anti–epidermal growth factor receptor, anti–transferrin receptor, and anti-CD22 antibodies, receptor expression is down-regulated after antibody binding. Thus, large amounts of anti-CD74 antibody, but not of the other internalizing mAbs, are transported into the target cells. Studies with other CD74-expressing cell types, including IFN-stimulated melanoma and colon carcinoma cell lines, supported the observations made in the lymphoma cells. In all the tested cell lines, CD74 internalized rapidly while being replaced by newly synthesized molecules, resulting in a high cumulative expression of CD74 on the surface, although the steady-state level was relatively low (40). Because the rapid internalization of LL1 provides rapid delivery of large numbers of molecules to lysosomes, it made LL1 an especially interesting candidate for the delivery of toxins, drugs, or radioisotopes that can kill CD74-expressing tumor cells.

Humanized LL1

A humanized form of LL1, hLL1, was generated by complementarity-determining region grafting and exhibited comparable antigen-binding and internalization properties as the parental murine antibody (30, 41). The humanized anti-CD74 mAb hLL1 causes specific growth inhibition and induction of apoptosis in B-cell lines in the presence of a second cross-linking antibody. In addition, significant survival extensions were observed in NHL- and MM-bearing severe combined immunodeficient (SCID) mice treated with naked hLL1 without the need for an exogenous cross-linking agent.

In comparisons of hLL1 to the chimeric anti-CD20 mAb, rituximab, we observed that the two antibodies act through distinct mechanisms and exhibit different expression and sensitivity profiles on B-cell malignancies (30, 42). As with rituximab, in most human lymphoma or MM cell lines, hLL1 alone does not show a direct cytotoxic effect *in vitro*. However, in the presence of an appropriate cross-linking agent, hLL1 causes inhibition of cell proliferation and induces apoptosis. Unlike rituximab, hLL1 induces little or no antibody-dependent cellular cytotoxicity or complement-mediated cytotoxicity. Antiproliferative effects of hLL1 and rituximab on a panel of NHL and MM cell lines are shown in Fig. 2. Incubation with hLL1 caused specific inhibition of proliferation in five of six NHL cell lines in the presence of a cross-linking second antibody (Fig. 2A). The sensitivity profile of the cell lines differed for rituximab (Fig. 2B). For example, in the absence of cross-linking, no antiproliferative activity was seen with hLL1 in any cell line, whereas rituximab yielded approximately 23% to 88% inhibition of Daudi, Ramos cells, and SU-DHL-6 cells. However, significant augmentation of the antiproliferative effects of rituximab was also observed with cross-linking. Interestingly, the variation in level of inhibition was not correlated strictly to antigen density. For example, in WSU-FSCCL, CD74 antigen density is half that of CD20; however, [^{3}H]thymidine incorporation was inhibited 80% by hLL1 + goat anti-human IgG cross-linking, whereas these cells were unaffected by rituximab + goat anti-human IgG. In MM cell lines, the antiproliferative activity of hLL1 and rituximab correlated with the presence of antigen in this cell line panel (Fig. 2C and D). ARH-77 and MC/CAR, CD20$^+$ and CD74$^+$ cell lines, were sensitive to both hLL1 and rituximab; CAG and

KMS12-PE, $CD20^-$, $CD74^+$, were only inhibited by hLL1; and the two $CD20^-$, $CD74^-$ cell lines, ARK and ARD, were insensitive to both mAbs. Importantly, antiproliferative activity can be augmented when anti-CD74 and anti-CD20 mAbs are combined (30).

In vivo, therapeutic efficacy of hLL1 was shown in SCID mice using NHL and MM cell lines. In the human Burkitt lymphoma xenografts, Daudi and Raji, median survival was extended significantly; a 45% increase was obtained in Raji and a 19% increase in Daudi-bearing SCID mice given lower doses. In both studies, treatments were initiated 1 day after injection of tumor cells (30). Therapeutic efficacy was markedly greater in the MC/CAR MM cell line. Figure 3 shows the results of a study comparing the efficacy of hLL1 in MC/CAR-bearing SCID mice treated by four dose schedules. Median survival of untreated mice was 28 days. The various treatment schedules initiated 5 days after tumor cell injection extended median survival to 49 to 112 days. Initiation of hLL1 dosing 1 day after tumor cells were inoculated yielded long-term survival (>150 days) in 8 of 10 mice. No treatment-related toxicities were observed as measured by body weight loss (30). Similar efficacy has recently also been observed in WSU-FSCCL follicular lymphoma and CAG MM xenografts, showing the broad usefulness of naked hLL1 against B-cell malignancies (data not shown).

The hLL1 safety profile was evaluated by doing toxicology studies in Cynomolgus monkeys because hLL1 cross-reacts with Cynomolgus monkey CD74. Up to 125 mg/kg were given in a single-dose escalation scheme and up to 50 mg/kg were given in a multidose escalation scheme (phase I: a single dose followed by a 4-week observation period; phase II: twice-weekly doses for 4 weeks). No test article-related mortality was observed at any dose. In addition, there was no overt evidence of systemic toxicity to any major organ, and clinical observations and clinical pathology were normal. Expected decreases were noted in peripheral blood mononuclear cells, including B and T lymphocytes, natural killer cells, and plasma cells, following the multidose scheme. None of the changes persisted through the 1- or 4-week recovery periods (Immunomedics, Inc.; data on file).

Radiolabeled Antibody

Although radioimmunotherapy has now been under investigation for over 2 decades, there has not been a consensus as to the choice of optimal radioisotope. Indeed, the choice of isotope depends on the specific application (tumor burden and tumor type), and specific tradeoffs between efficacy and toxicity must be made. The high-energy β-emitters, such as ^{131}I and ^{90}Y, have been widely used to kill large tumor masses. However, the radiation dose delivered is primarily due to crossfire from neighboring cells and can cause significant toxicity, especially in bone marrow. In addition, such radiation is unable to efficiently kill single cells. This is significant clinically because it implies that mAbs carrying ^{131}I and ^{90}Y cannot efficiently kill micrometastases or single tumor cells. α-Particles can kill single cells effectively due to their relatively short path length and high energy. However, most available α-particle emitters have short half-lives (≤7 h) and are therefore unsuitable for treatment of solid tumors because of the substantial time required for tumor penetration but may be of value for micrometastatic disease (43). ^{225}Ac is a long half-life α-emitter that has been used in humans, but it is expensive and difficult to obtain. Auger electron-emitting radionuclides are a third category of toxic isotopes. The low energy of the emissions requires the isotope to be in close proximity to the nucleus or present at high concentration. When these conditions are met, Auger electron-emitting radionuclides are suitable for killing single cells and micrometastases.

LL1 has been evaluated with all three of these types of radioisotopes. The unique property of LL1 is its high level of intracellular uptake. Because of this, conjugates of Auger electrons and LL1 can be used to deliver a greater quantity of Auger electrons than other conjugated mAbs. *In vitro*, B-lymphoma cells are efficiently and specifically killed by LL1 conjugated to various radiolabels, including ^{111}In, ^{125}I, ^{99m}Tc, ^{67}Ga, ^{131}I, and ^{90}Y (44, 45). Total killing was obtained (>6 logs) under conditions in which a nonreactive antibody labeled in the same way produced no significant toxicity. Because of the rapid internalization and catabolism of LL1, radioiodine conjugates were only active if "residualizing" forms were used (i.e., forms that are trapped inside the cell after catabolism of the antibody). Conventional oxidative iodination produces a nonresidualizing label in which the radioiodotyrosine produced after internalization and catabolism of labeled mAb diffuses from the target cell precluding accumulation of isotope (45). Conjugates of LL1 with the β-particle emitters yielded more nonspecific toxicity in *in vitro* studies than Auger emitters, and correspondingly, much lower doses of β-particle–emitting isotopes could be given in an *in vivo* model of tumor growth.

A mouse xenograft model, consisting of Raji human B-cell lymphoma cells injected i.v. into SCID mice, was used to determine whether specific therapeutic effects could be shown *in vivo* with LL1 conjugates of ^{111}In, ^{67}Ga, and ^{90}Y (46). Radiolabeled mAbs were injected at various times after tumor inoculation. Tumor growth was monitored by hind leg paralysis. With a 3- to 5-day interval before antibody injection, LL1 conjugated to the Auger emitters (^{111}In and ^{67}Ga) at a dose of 240 to 350 μCi/mouse yielded a strong therapeutic

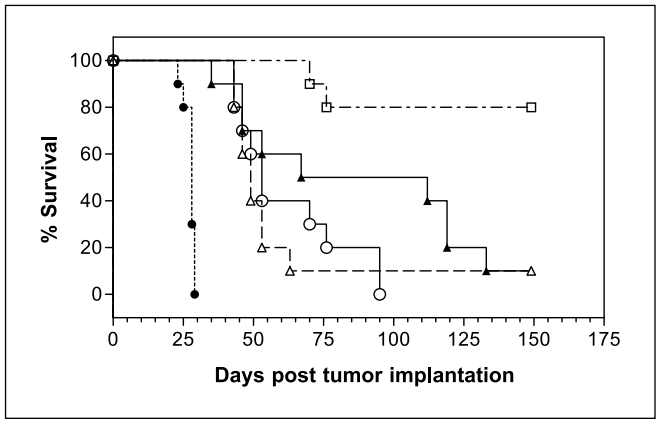

Fig. 3. Therapy of disseminated MM xenografts in SCID mice with hLL1. mAb hLL1 was given to MC/CAR-bearing SCID mice at four dose schedules. Survival was compared with untreated MC/CAR-bearing SCID mice. Δ, 350 μg given 5 d after tumor cells were grafted; ○, 350 μg/injection once weekly for 2 wks, treatment initiated 5 d after tumor cells were injected; ▲, 100 μg/injection five times weekly for 2 wks, then twice weekly, treatment initiated 5 d after tumor cells were inoculated; □, 100 μg/injection five times weekly for 2 wks, then twice weekly, treatment initiated 1 d after tumor cells were injected; ●, untreated control. Reproduced with permission from Stein et al. (30).

effect, with greatly delayed tumor growth. Moreover, many of the treated mice were tumor-free for >6 months, whereas control mice became paralyzed in 16 to 24 days. With ^{90}Y-labeled LL1, the maximal tolerated dose was markedly lower, 25 μCi/mouse, and at this dose there was only a weak therapeutic effect. Effective and specific therapy of disseminated B-cell lymphoma in SCID mice with ^{111}In-labeled LL1 is shown in Fig. 4A. ^{111}In-LL1 increased median survival ~7-fold, from 18 to 124 days, and produced a complete cure (6 months without evidence of tumor growth) in 40% of the mice. A dose-response experiment with ^{111}In-LL1 is shown in Fig. 4B, where treatment was given 5 days after tumor cells in this study. Median survivals of 19, 28, 40, and 115.5 days were observed, respectively, in the untreated and 31 μCi, 92 μCi, and 275 μCi ^{111}In-LL1 treatment groups. Experiments in which the interval between tumor injection and mAb administration was varied are shown in Fig. 4C. During the 6-month observation period, only 1 of 10 mice died (at day 155) when 350 μCi ^{111}In-LL1 was given 3 days after cells.

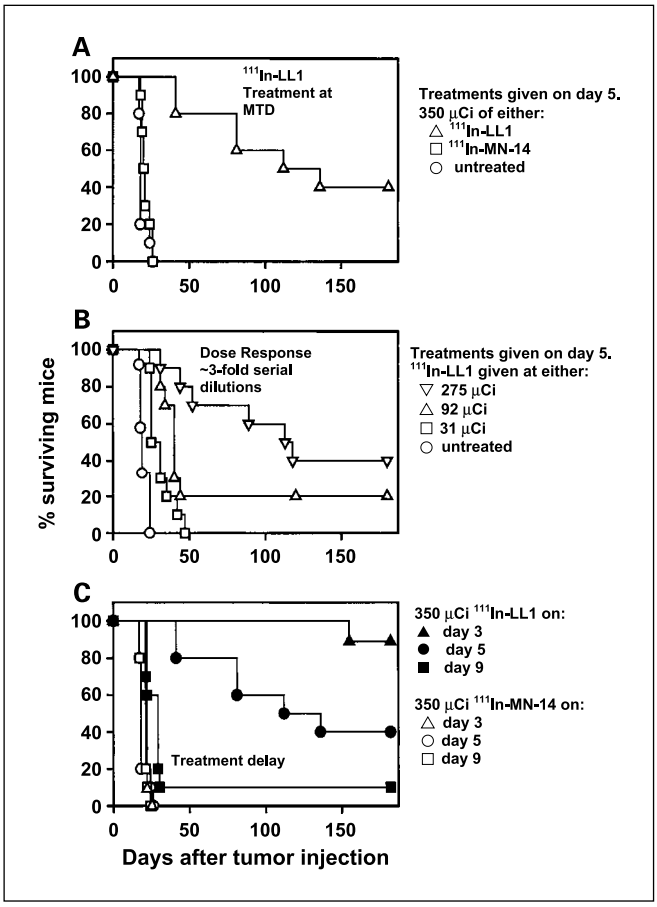

Fig. 4. Therapy of disseminated Raji xenografts in SCID mice with ^{111}In-labeled LL1. *A,* 5 d after i.v. injection of tumor cells, groups of 10 mice were injected with 350 μCi of either ^{111}In-labeled LL1 (Δ) or ^{111}In-labeled MN-14, an anti – carcinoembryonic antigen mAb, unreactive with Raji (□). ○, a control group of 10 mice had no antibody injected. MTD, maximum tolerated dose. *B,* groups of 10 mice were treated on day 5 with 275 μCi (▽), 92 μCi (Δ), or 31 μCi (□) of ^{111}In-labeled LL1. ○, results with 12 control untreated mice. *C,* the time of antibody injection was day 3 (*triangles*), day 5 (*circles*), or day 9 (*squares*) after tumor cell injection, with a dose of 350 μCi of ^{111}In-labeled LL1. Results with untreated control mice (groups of 10-11 mice, *open symbols*) and antibody treated mice (groups of 10 mice, *filled symbols*). Reproduced with permission from Ochakovskaya et al. (46).

Median survivals of 124 and 29 days were obtained with 5- and 9-day intervals, respectively. No significant toxicity was observed. Unlabeled LL1 given at the same concentration and dose schedule as the radiolabeled preparations did not have a significant effect on survival. Thus, under these conditions, the therapeutic effects observed were attributable to the radioactivity and not the mAb itself. Moreover, a similarly strong therapeutic effect was observed using the maximum tolerated dose (240 μCi/mouse) of ^{67}Ga-LL1. In contrast, ^{90}Y-labeled LL1 used at the maximum tolerated dose (25 μCi/mouse) had only a small therapeutic effect; the median survival time was increased only from 18 to 29 days. ^{111}In-LL1 was also observed to be an effective therapeutic agent in a study of small s.c. B-lymphoma xenografts, extending the utility of this agent to a somewhat larger tumor size (47).

The α-emitter ^{213}Bi, with a half-life of 46 min, was conjugated to LL1 with the chelator CHX-A"-DTPA. The conjugate was an effective and specific toxic agent for B-cell lymphoma both *in vitro* and *in vivo* in the SCID mouse Raji cell xenograft micrometastatic model (43). Although the short half-life limits the usefulness of ^{213}Bi-mAb conjugates for solid tumors, they may be useful for treatment of accessible tumors, such as leukemia cells or micrometastases, where antibody binding is rapid. In addition, α-particle emitters may be useful with antibody fragments due to the faster tumor penetration of the lower molecular weight fragments. Progress in the use of α-particle emitters has been made and there are ongoing studies that may bring additional enthusiasm to their use in radioimmunotherapy (48, 49).

These observations show that LL1 is an effective agent for delivery of radionuclides, including both Auger (^{111}In, ^{67}Ga, and ^{125}I) and α-particle (^{213}Bi) emitters. ^{111}In- and ^{67}Ga-labeled LL1 were markedly superior to ^{90}Y-LL1, suggesting that these isotopes are more effective than conjugates with β-particle emitters for the treatment of micrometastases. This was corroborated in a recent study of ^{177}Lu-LL1 (50). The potency of LL1 labeled with Auger electron-emitting radioisotopes is due to the remarkably high level of accumulation within cells.

Drug and Toxin Antibody Conjugates

Conjugates of mAbs with drugs or toxins have been investigated for many years as a potential approach to delivering these agents more specifically to cancers (51). Although it was contended previously that conventional chemotherapy drugs, such as doxorubicin (dox), had inadequate potency to elicit a clinical effect when conjugated to a mAb (52–55), the rapid internalization of LL1 led us to reevaluate this concept using this antibody. The chemical properties of dox and similar anthracyclines are favorable for protein linkage. Specifically, anthracyclines are water soluble, not too hydrophobic, and have several reactive groups usable as linkage sites. Finally, dox is a known chemotherapeutic, acting by several mechanisms, including inhibition of topoisomerase II, intercalation into DNA, and effects on cell membranes (56, 57).

hLL1-dox (IMMU-110) is a drug immunoconjugate composed of dox conjugated to hLL1 at 6 to 8 drug molecules per IgG molecule. The dox is linked to partially reduced antibody interchain disulfide groups using 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylhydrazide. Evaluation of hLL1-dox and

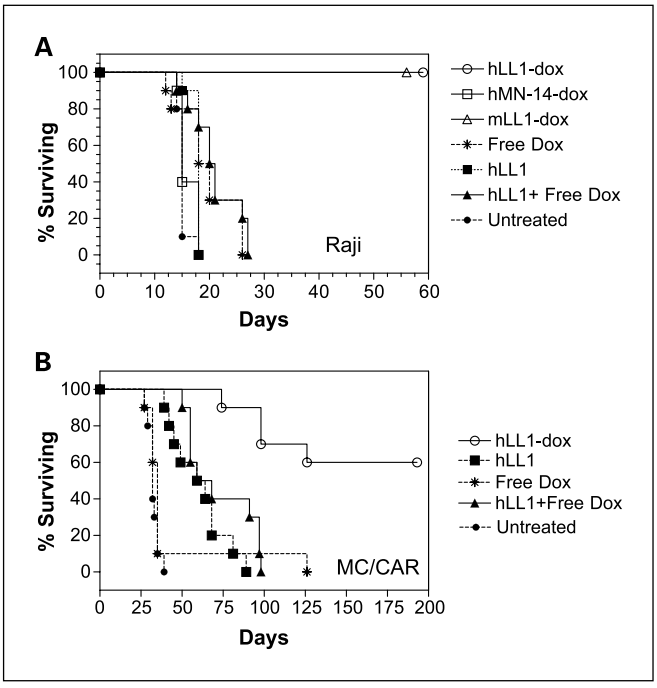

Fig. 5. Therapy of disseminated NHL and MM xenografts in SCID mice with hLL1-dox. SCID mice (8-10 per group) were injected i.v. with 2.5×10^6 Raji NHL cells (*A*) or 1×10^7 MC/CAR MM cells (*B*). After 5 d, mice were treated with single i.v. doses of dox conjugates of hLL1 (○) and murine LL1 (Δ) in comparison with control groups, including animals given the same dose of free dox (□), unconjugated hLL1 alone (■), a mixture of free dox + unconjugated hLL1 (▲), untreated (●), and a nonreactive conjugate, hMN-14-dox (□). *A,* reproduced with permission from Griffiths et al. (60). *B,* reproduced with permission from Sapra et al. (61).

murine LL1-dox in cell lines and tumor-bearing SCID mice showed very high antitumor activity in both NHL and MM models. Toxicity of hLL1-dox was similar to that of free dox in $CD74^+$ cell lines, whereas the control conjugate was markedly less toxic (58, 59). In $CD74^-$ cell lines, toxicity of hLL1-dox was similar to the negative control conjugate. *In vivo* efficacy is shown in Fig. 5. Results of single-dose treatments given 5 days after administration of tumor cells are shown in Fig. 5A for a NHL model (Raji) and Fig. 5B for a MM model (MC/CAR). At 350 μg/animal, hLL1-dox led to long-term survival of 100% of Raji-bearing mice and 60% of MC/CAR-bearing mice, whereas no significant effects were seen with unconjugated dox given at an equivalent dose or control conjugates (60, 61). The naked hLL1 had significant antitumor effects in the MM model but less so than the drug immunoconjugate. Similar survival extensions were observed when treatment with hLL1-dox was given up to 10 days after transplantation of MM or NHL cells, and significant, but smaller, survival extension was even observed 14 days after tumor cell inoculation. The efficacy of hLL1-dox was confirmed in other studies in SCID mice bearing the Burkitt lymphoma Daudi (61) and the follicular lymphoma WSU-FSCCL (data not shown).

In SCID mice, hLL1-dox failed to show any toxicity, including myelotoxicity and cardiotoxicity, up to the maximum single dose tested of 2.5 mg/mouse (protein dose = 125 mg/kg; dox equivalent dose = 3.6 mg/kg), although the maximum tolerated dose of free dox was reached at 3.6 mg/kg. Therefore, conjugation of dox to hLL1 mitigated the toxicity of free dox in SCID mice. Lack of cross-reactivity with rodent CD74 makes safety studies in mice less relevant. Therefore, preliminary tolerability studies of hLL1-dox in nonhuman primates (cynomolgus monkeys) were done because hLL1 reacts with monkey CD74. Although these acute toxicity studies were done in a relatively small number of animals, they suggest that hLL1-dox is well tolerated up to 30 mg/kg, at which level the first signs of bone marrow toxicity appeared. No acute cardiotoxicity or signs of adverse effects to other major organs were observed at doses up to 90 mg/kg (61).

An excellent therapeutic index was also achieved in preclinical models of NHL with a recombinant fusion protein of humanized anti-CD74 and the toxin Ranpirnase (Rap; ref. 62). Rap is an amphibian RNase belonging to the RNase A superfamily (63). Rap has a low affinity for the RNase inhibitor present at high levels in mammalian cells and therefore evades inactivation. Rap enters cells via receptor-mediated endocytosis and, once internalized, selectively degrades tRNA, resulting in inhibition of protein synthesis and induction of apoptosis (64–66). A novel recombinant RNase-anti-CD74 humanized IgG4 antibody immunotoxin, 2L-Rap-hLL1-γ4P, composed of 2 Rap molecules, each fused to the NH_2 terminus of the light chain of hLL1, was designed and expressed. To reduce unwanted side effects caused by γ1 effector cell functions, the constant region of hLL1 was changed from γ1 to γ4. In addition, a point mutation, $Ser^{241}Pro$, was introduced into the hinge region of the γ4 sequence to avoid formation of half-molecules when the antibody is expressed and produced in mammalian cell cultures (67). *In vitro*, 2L-Rap-hLL1-γ4P retained RNase activity and specific binding to CD74 and was significantly more potent against NHL and MM $CD74^+$ cell lines than naked hLL1. *In vivo*, the pharmacokinetic profile of 2L-Rap-hLL1-γ4P was similar to that of hLL1. Most importantly, marked therapeutic efficacy was shown in animal models of

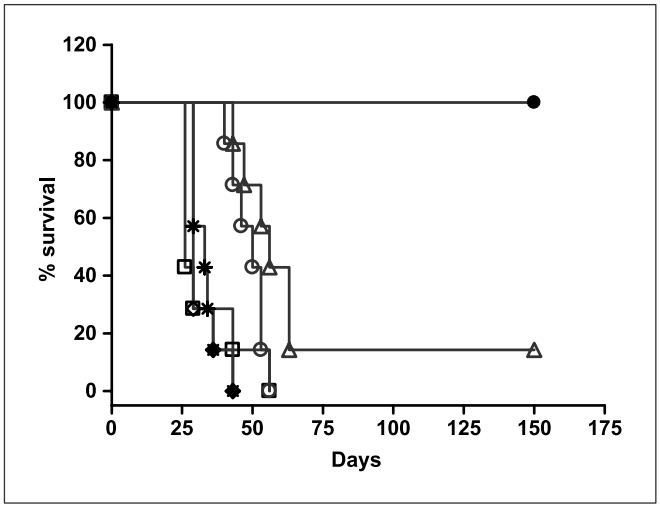

Fig. 6. Therapy of disseminated Daudi NHL human xenograft with 2L-Rap-hLL1-γ4P. SCID mice (seven to eight per group) were injected i.v. with 1.5×10^7 Daudi cells. ●, after 1 d, mice were treated with a single bolus i.v. injection of 15 μg 2L-Rap-hLL1-γ4P. Control animals received saline (□), 13 μg naked hLL1 (○), 2 μg rRap (◇), a mixture of naked hLL1 and rRap (Δ), and a nonspecific immunotoxin, 2L-Rap-hRS7 (□). Reproduced with permission from Chang et al. (62).

B-cell lymphomas (Daudi and Raji), where treatment with a single 5 to 50 μg dose of 2L-Rap-hLL1-γ4P, given in early or advanced disease, resulted in cures of most animals (62).

Figure 6 summarizes a study in which Rap-hLL1 specifically targeted Daudi xenografts and yielded long-term survival in all treated mice. SCID mice were injected i.v. with Daudi cells, and treatment was initiated 1 day after injection of the tumor cells. A single dose of 15 μg of 2L-Rap-hLL1-γ4P yielded cures in 100% of the animals. Treatment with 2 μg rRap (corresponding to the amount in 15 μg 2L-Rap-hLL1-γ4P) or 50 μg of nonspecific immunotoxin (2L-Rap-hRS7) had no therapeutic effect. Treatment with 13 μg naked hLL1-γ4P or with a mixture of 13 μg naked hLL1-γ4P and 2 μg rRap led to an approximate doubling of the median survival times observed in mice injected with saline but was significantly less effective than 2L-Rap-hLL1-γ4P (62).

Immunogenicity, vascular leak syndrome, and nonspecific hepatotoxicity are among major concerns of immunotoxins constructed with plant and bacterial toxins (68, 69). Because the native sequence of Rap lacks the amino acid sequence motif that is responsible for binding to endothelial cells (70), vascular leak syndrome may be less of a concern for Rap-containing immunotoxins. Evaluations of this question as well as of potential immunogenicity of 2L-Rap-hLL1-γ4P are required. Toxicity studies of 2L-Rap-hLL1-γ4P in mouse models showed a nonspecific hepatotoxicity of the immunotoxin when elevated doses were given. This is not unexpected because the Rap-hIgG fusion proteins are highly basic (isoelectric point above 10) and earlier work with *Pseudomonas* immunotoxin had shown that increased liver toxicity could be correlated with higher isoelectric point values. The excellent therapeutic index of 2L-Rap-hLL1-γ4P in xenograft models (~50-fold above hepatotoxic doses), together with the successful clinical studies using *Pseudomonas* immunotoxin (71), suggest that clinical studies of 2L-Rap-hLL1-γ4P in lymphomas and myelomas are warranted. In addition to the high therapeutic index of 2L-Rap-hLL1-γ4P, recombinant immunotoxins produced as reported by Chang et al. (62) have additional practical advantages, including simple and scalable purification processes, homogeneous and fully functional products, and comparable yields to those of humanized antibodies.

Conclusions

The biological function of CD74 is just beginning to be appreciated in normal and malignant cells, where it seems to serve as a signaling molecule in cell proliferation and survival as well as having a potential role in innate and adaptive immunity. These functions are being elucidated by the use of CD74 activators and antagonists, including specific antibodies, such as the LL1 antagonistic antibody developed by our group. Preclinical studies of the humanized anti-CD74 mAb hLL1 have shown that it is an effective therapeutic agent that may be of significant value for treatment of B-cell malignancies, such as NHL and MM. To the best of our knowledge, these studies were the first to suggest that CD74 antagonistic antibodies could be potential therapeutic agents for the treatment of CD74-expressing malignancies. Application of hLL1 should be examined as a single-agent and also as part of multiple-agent therapies. Moreover, hLL1 may be especially useful as a carrier of therapeutic drugs, radioisotopes, or toxins due to internalization and rapid turnover of the CD74 antigen when bound with this antibody. In light of the expression of CD74 in certain nonhematologic malignancies, the potential of hLL1 as therapy for these diseases also should be evaluated. Development of hLL1 for clinical use is currently in progress.

In addition to the development of hLL1 as a potential therapeutic, the work described in this review has resulted in observations and methodologies that may be applicable in other systems. Specifically, the approach to the development of recombinant immunotoxins may be applied to the construction of a variety of immunoconjugates containing different toxin moieties and antigen-specific antibodies. In addition, the demonstration that Auger-emitting radioisotopes, such as ^{111}In, and α-particle–emitting radionuclides, such as ^{213}Bi, can be used effectively for radioimmunotherapy with an appropriate mAb has advanced the field of radioimmunotherapy by suggesting that these may be advantageous in the treatment of dispersed, single-cell, or oligocellular disease, such as leukemias or micrometastatic cancers.

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