A Bispecific Antibody-IFNa2b Immunocytokine Targeting CD20 and HLA-DR Is Highly Toxic to Human Lymphoma and Multiple Myeloma Cells

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Abstract

The short circulating half-life and side effects of IFNα affect its dosing schedule and efficacy. Fusion of IFNα to a tumor-targeting monoclonal antibody (MAb-IFNα) can enhance potency due to increased tumor localization and improved pharmacokinetics. We report the generation and characterization of the first bispecific MAb-IFNα, designated 20-C2-2b, which comprises two copies of IFNα2b and a stabilized F(ab)2 of hL243 (humanized anti–HLA-DR; IMMU-114) site-specifically linked to veltuzumab (humanized anti-CD20). In vitro, 20-C2-2b inhibited each of four lymphoma and eight myeloma cell lines, and was more effective than monospecific CD20-targeted MAb-IFNα or a mixture comprising the parental antibodies and IFNα in all but one (HLA-DR+/CD20−) myeloma line, suggesting that 20-C2-2b should be useful in the treatment of various hematopoietic malignancies. 20-C2-2b displayed greater cytotoxicity against KMS12-BM (CD20+/HLA-DR+ myeloma) compared with monospecific MAb-IFNα, which targets only HLA-DR or CD20, indicating that all three components in 20-C2-2b could contribute to toxicity. Our findings indicate that a given cell's responsiveness to MAb-IFNα depends on its sensitivity to IFNα and the specific antibodies, as well as the expression and density of the targeted antigens. Cancer Res; 70(19); 7600−9. ©2010 AACR.

Introduction

In the United States, there were 65,980 new cases of non-Hodgkin lymphoma (NHL) and 19,500 deaths from this disease in 2009 (1). Approximately half of patients with NHL fail first-line therapy and are rarely cured (2). In addition to NHL, there were 20,580 new cases and 10,580 deaths from multiple myeloma (MM; ref. 1). The clinical activity of IFNα is established in NHL therapy (3, 4), and the addition of IFNα to rituximab immunotherapy has shown some clinical advantage (5, 6). Available data suggest that progression-free survival of MM patients is improved with IFNα, but the benefit is small and its use remains controversial because of toxicity (7). Even though IFNα can have direct cytotoxic activity on tumors, inhibit angiogenesis, and stimulate both innate and adaptive immunity, its efficacy as an anticancer therapeutic has been limited due to its short circulating half-life and systemic toxicity. Selective delivery using tumor-targeting monoclonal antibodies (MAb) could improve pharmacokinetics, increase local concentration, prolong tumor retention, and limit systemic exposure of IFNα, thereby augmenting its direct and indirect effects and increasing the therapeutic index.

The Dock-and-Lock (DNL) method is a modular approach for building multivalent, multispecific, and multifunctional agents suitable for biomedical applications (8, 9). Previously, using DNL, we generated a novel immunocytokine named 20-2b-2b, formerly 20-2b, which comprises four IFNα2b groups tethered to the humanized anti-CD20 MAb, veltuzumab (v-mab), and showed potent in vitro cytotoxicity and superior therapeutic efficacy in human NHL xenograft models (10). This inspired our hypothesis that an immunocytokine that targets CD20 and a second highly expressed antigen on such malignant cells could be even more efficacious.

One such target, HLA-DR, is expressed on many hematopoietic malignancies. IMMU-114 (or hL243γ4p; ref. 11), which shows potent direct cytotoxicity on NHL in vitro and in vivo, is a humanized IgG4 version of L243, a mouse anti–HLA-DR MAb, which was engineered to prevent the formation of half-IgG associated with the IgG4 isotype (12). As an IgG4 variant, the effector functions of IMMU-114, particularly complement-dependent cytotoxicity (CDC), are minimized (11).

Herein, we describe the first bispecific MAb-IFNα (20-C2-2b), which was generated using the DNL method to comprise v-mab fused to a stabilized hL243 F(ab)2 and dimeric IFNα2b. We show that 20-C2-2b has potent cytotoxicity against four NHL cell lines as well as eight MM cell lines, seven of which express HLA-DR. Because 20-C2-2b has antibody-dependent cellular cytotoxicity (ADCC), but not CDC, and can target both CD20 and HLA-DR, it may be useful for therapy of a
broad range of hematopoietic cancers that express either or both antigens. The bispecific immunocytokine may be particularly effective in the elimination of the putative cancer stem cells associated with myeloma, which are resistant to current therapy regimens and reportedly express CD20.

Materials and Methods

Antibodies and cell culture
The following MAbs were provided by Immunomedics, Inc.: veltuzumab or v-mab (anti-CD20 IgG1), hL243γ4p (Immu-114, anti-HLA-DR IgG4), a murine anti-IFNα MAb, and rat anti-idiotype MAbs to v-mab (WR2) and hL243 (WT). Heat-inactivated fetal bovine serum was obtained from Hyclone. All other cell culture media and supplements were purchased from Invitrogen Life Technologies. Codes and descriptions of the MAbs, DNL modules, and reagent mixtures used in this study are provided in Table 1B and C. Sp/ESF cells, a cell line derived from Sp2/0 with superior growth properties,

Figure 1. Schematics of 20-C2-2b and its constituent DNL modules. A, structures and expression cassettes of C3-AD2-lgG-v-mab (20), C31-DDD2-Fab-hL243 (C2), IFNα2b-DDD2 (2b), and 20-C2-2b. Blue and red helices represent DDD2 and AD2, respectively; SH, sulfhydryl groups of engineered cysteines; v-mab V, variable (green); hL243 V (purple); C, constant (gray); G, hinge; L, linker; H, 6-His-linker. B, description of codes for DNL modules, MAbs, and reagent mixtures used in this study. C, key describing DNL conjugates used in this study.

4 Rossi DL, unpublished results.
pyruvate, 10 mmol/L of l-glutamine, and 25 mmol/L of HEPES. Daudi, Ramos, Raji, Jeko-1, NCI-H929, and U266 human lymphoma cell lines were purchased from American Type Culture Collection. The sources of MM cell lines are as follows: KMS11, KMS12-S, and KMS12-BM from Dr. Takemi Otsuki (Kawasaki Medical School, Okayama, Japan); CAG, OPM-6, and MM.1R from Dr. Joshua Epstein (University of Arkansas, Little Rock, AK), Dr. Kenji Oritani (Osaka University, Osaka, Japan), and Dr. Steven Rosen (Northwestern University, Chicago, IL), respectively. All cell lines were authenticated by the supplier, obtained within 6 months of their use, and passaged less than 50 times. We did not re-authenticate the cell lines.

**DNL constructs**

Monospecific MAB-IFNox (20-2b-2b, 734-2b-2b and C2-2b-2b) and the bispecific HexAb (20-C2-C2) was generated by combination of an IgG-AD2 module with DDD2 modules using the DNL method (Fig. 1C), as described previously (10, 13). The 734-2b-2b, which comprises tetrameric IFNox2b and MAb h734 (anti-indium DTPIA IgG1), was used as a nontargeting control MAB-IFNox.

The bispecific MAB-IFNox (20-C2-2b) was prepared as follows. Briefly, three DNL modules (C93-AD2-lgG-v-mab, C91-DDD2-Fab-hL243, and IFNox2b-DDD2) were combined in equimolar quantities and incubated overnight with 1 mmol/L of reduced glutathione before the addition of oxidized glutathione (2 mmol/L). 20-C2-2b was purified sequentially on MAbSelect (GE Healthcare), His-Select (Sigma-Aldrich), and immobilized WT affinity columns.

**Analytic methods**

Size exclusion high-performance liquid chromatography (SE-HPLC) was performed using an Alliance HPLC System with a BioSuite 250, 4 μm of UHR SEC column (Waters Corp.). Immunoreactivity was assessed by mixing excess WT, anti-IFNox, or WR2 with 20-C2-2b prior to analysis of the resulting immune complex by SE-HPLC.

SDS-PAGE was performed under reducing and nonreducing conditions using 12% and 4% to 20% gradient Tris-glycine gels (Invitrogen), respectively. Electrospray ionization time of flight liquid chromatography/mass spectrometry was performed with a 1200 series HPLC coupled with a 6210 time of flight mass spectrometry (Agilent Technologies). 20-C2-2b was reduced with 10 mmol/L of tris(2-carboxyethyl)phosphine at 60°C for 30 minutes and resolved by SDS-PAGE, the capillary and fragmentor voltages were set to 5,500 and 200 V, respectively.

IFNox2b-specific activities were determined using the iLite Human Interferon Alpha Cell-Based Assay Kit (PBL Interferon Source), as described previously (10). Peginterferon alfa-2b (Schering Corp.) was used as a positive control.

**Cell binding and apoptosis**

Cell binding and apoptosis were assessed by flow cytometry using a Guava PCA and the reagents, software, and suggested protocols for Guava Express and Guava Nexin, respectively (Millipore).

For binding assays, live cells were incubated for 1 hour at 4°C with MAbs or MAB-IFNox diluted in 1% BSA-PBS. Cells were pelleted and washed twice with 1% BSA-PBS before incubation for 1 hour at 4°C with 2 μg/mL of PE-conjugated mouse antihuman IgG-Fc (Southern Biotech). After three washes, binding was measured by flow cytometry.

For apoptosis assays, cells (5 × 10⁵/mL) were incubated with the indicated MAb or MAB-IFNox in 24-well plates for 48 hours before quantification of the percentage of Annexin V-positive cells.

**In vitro cytotoxicity**

Cells were seeded in 48-well plates (300 μL/well) at predetermined optimal initial densities (1–2.5 × 10⁵ cells/mL) in the presence of increasing concentrations of the indicated agents and incubated at 37°C until the density of untreated cells increased ≥10-fold (4–7 d). Relative viable cell densities at the end of the assay were determined using a CellTiter 96 Cell Proliferation Assay (Promega).

**Ex vivo depletion of Daudi from whole blood**

Blood specimens were collected under a protocol approved by the New England Institutional Review Board (Wellesley, MA). Daudi (5 × 10⁵) cells were mixed with heparinized whole blood (150 μL) from healthy volunteers and incubated with MAbs or MAB-IFNox at 1 mmol/L for 2 days at 37°C and 5% CO₂. Cells were stained with FITC anti-CD19, FITC anti-CD14, APC anti-CD3, or APC mouse IgG1 isotype control MAb (BD Biosciences) and analyzed by flow cytometry using a FACS Calibur (BD Biosciences). Daudi cells are CD19+ and in the monocyte gate. Normal B and T cells are CD19+ and CD3+ cells, respectively, in the lymphocyte gate. Monocytes are CD14+ cells in the monocyte gate.

**Results**

**Generation and characterization of 20-C2-2b**

The bispecific MAB-IFNox was generated by combining the IgG-AD2 module, C93-AD2-lgG-v-mab, with two different dimeric DDD modules, C91-DDD2-Fab-hL243 and IFNox2b-DDD2 (Fig. 1A). Due to the random association of either DDD module with the two AD2 groups, two side-products, 20-C2-C2 and 20-2b-2b are expected to form, in addition to 20-C2-2b (Fig. 1C).

A detailed description of the generation, purification, and biochemical characterization of 20-C2-2b is provided in the Supplementary Results. The desired product (20-C2-2b) was purified from the mixture using MABSelect, His-Select, and immobilized WT, which sequentially removed unreacted DDD2 modules, 20-C2-C2, and 20-2b-2b, respectively. The purity of 20-C2-2b was shown by SDS-PAGE, which resolved a cluster of high relative mobility (Mᵣ) bands of the appropriate molecular size and the five polypeptides comprising 20-C2-2b under nonreducing and reducing conditions, respectively. (Supplementary Fig. S1A and B). The mass of each of the polypeptides comprising 20-C2-2b determined by liquid
chromatography/mass spectrometry was consistent with the mass calculated from their deduced amino acid sequence and predicted posttranslational modifications, including N-linked glycosylation and amino-terminal pyroglutamate on the HC-AD2 polypeptide as well as O-linked glycosylation on a portion of the IFNα2b-DDD2 (ref. 14; Supplementary Fig. S1C, Supplementary Table S1).

SE-HPLC analysis of 20-C2-2b resolved a predominant protein peak with a retention time (6.7 min) consistent with its calculated mass and between those of the larger 20-C2-C2 (6.6 min) and smaller 20-2b-2b (6.85 min), as well as some higher molecular weight peaks that likely represent noncovalent dimers formed via self-association of IFNα2b (Supplementary Fig. S1D).

Immunoreactivity assays showed the homogeneity of 20-C2-2b with each molecule containing the three functional groups (Supplementary Fig. S1E). Incubation of 20-C2-2b with an excess of antibodies to any of the three constituent modules resulted in quantitative formation of high molecular weight immune complexes and the disappearance of the 20-C2-2b peak. The His-Select and WT affinity unbound fractions were not immunoreactive with WT and anti-IFNα, respectively (data not shown).

Cell binding

The MAb-IFNα showed similar binding avidity to the parental MAbs (Fig. 2A). At subsaturating concentrations, similar binding levels were observed for 20-C2-2b and hL243γ4p. The antigen density of HLA-DR is ∼6-fold greater than CD20 in these cells, allowing more binding of 20-C2-2b compared with 20-2b-2b (15). Binding curves, which were analyzed using a one-site binding nonlinear regression model, showed that 20-C2-2b could achieve a 4.7-fold higher Bmax compared with 20-2b-2b, with no significant difference observed between their binding affinities (Kd ∼4 nmol/L; Fig. 2B).

IFNα biological activity

The specific activities for various MAb-IFNα were measured using a cell-based reporter gene assay and compared with peginterferon alfa-2b (Fig. 2C). Expectedly, the specific activity of 20-C2-2b (2,454 IU/pmol), which has two IFNα2b groups, was significantly lower than that of 20-2b-2b (4,447 IU/pmol) or 734-2b-2b (3,764 IU/pmol), yet greater than peginterferon alfa-2b (P < 0.001). The difference between 20-2b-2b and 734-2b-2b was not significant. The specific activity among all agents varies minimally when normalized to IU/pmol of total IFNα. Based on these data, the specific activity of each IFNα2b group of the MAb-IFNα is approximately 30% of recombinant IFNα2b (∼4,000 IU/pmol).

In vitro cytotoxicity: NHL

The results of in vitro cytotoxicity assays with B-cell NHL are summarized in Table 1, with individual dose–response curves shown in Supplementary Fig. S4. The relative antigen densities of HLA-DR and CD20 for each cell line have been reported (15). The targeting index (TI) represents the fold increase in potency of a targeted MAb-IFNα compared with nontargeted MAb-IFNα (734-2b-2b), with the EC50 values converted to total IFNα concentration (I-EC50). Daudi is very sensitive to cell killing by IFNNo2, as shown with the nontargeting MAb-IFNα, 734-2b-2b (I-EC50 = 14 pmol/L). Targeting CD20 on Daudi with the monospecific 20-2b-2b (I-EC50 = 0.4 pmol/L) further enhanced the potency 25-fold (TI = 25), consistent with previous results (10). The potency enhancement for the bispecific 20-C2-2b (I-EC50 = 0.08 pmol/L; TI = 125) was 5-fold greater than 20-2b-2b, which can be attributed...
to the added antigen density of HLA-DR and possibly its high-avidity tetravalent tumor binding. It is less likely that hL243-induced signaling contributes additional cytotoxicity at these low concentrations. The mixture of v-mab, hL243γ4p, and 734-2b-2b (v-mab + hL243 + 734-2b) was equal to 734-2b-2b alone, supporting the conclusion that hL243-induced signaling does not contribute to the high TI of 20-C2-2b. Apoptosis was induced in Daudi with only 1 pmol/L of any MAb-IFNγ, whereas the mixture comprising hL243 was superior to 20-C2-2b (EC50 = 1 nmol/L). 20-C2-2b exhibited 2-fold enhanced potency compared with hL243γ4p or v-mab + hL243 + 734-2b (<0.0005). There was no significant difference observed between 734-2b-2b and the mixture.

The 734-2b-2b had less effect on Raji (I-EC50 = 32 nmol/L) and Ramos (I-EC50 > 80 nmol/L), resulting in maximal inhibition (lmax) of only 62% and 35%, respectively. Under these conditions, hL243γ4p, but not v-mab (data not shown), inhibited these Burkitt lymphoma lines. The observed enhancement in potency of 20-C2-2b (TI = 118) was >50-fold greater than 20-C2-2b for Raji, which has much greater HLA-DR density, was inhibited by hL243 but not v-mab (data not shown). Targeting the IFNγ with only 1 pmol/L of any MAb-IFNγ does not contribute to the high TI of 20-C2-2b. Apoptosis was induced in Daudi with only 1 pmol/L of any MAb-IFNγ but not with 10 pmol/L of v-mab or hL243γ4p (Fig. 3A). Treatment with 20-C2-2b or 20-C2-2b resulted in significantly more apoptotic cells than 734-2b-2b or v-mab + hL243γ4p (P < 0.0005). The mantle cell lymphoma, Jeko-1, was considerably more responsive to IFNγ than either agent alone (Fig. 3A). Presumably, v-mab has little contribution in the mixture because alone it had only a modest effect. Both 20-C2-2b and the mixture were superior to 20-C2-2b (P < 0.002), due to the action of hL243.

**In vitro cytotoxicity: myeloma**

Whereas the eight MM cell lines vary in HLA-DR levels (and only KMS12-BM expresses CD20) and sensitivity to IFNγ (Fig. 4), all responded to 20-C2-2b. Dose–response curves for each of the eight MM cell lines tested are shown in Fig. 5, and the results are summarized in Table 1. For example, five were highly responsive to IFNγ (I-EC50 < 1 nmol/L for 734-2b-2b), but varied in HLA-DR antigen density. Of these, only CAG, which has high HLA-DR density, was inhibited by hL243γ4p (>1 nmol/L), and showed an increased (additive) response to a mixture of hL243γ4p and 734-2b-2b (hL243 + 734-2b) at higher

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### Table 1. *In vitro* cytotoxicity of MAb-IFNγ on NHL and MM cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>20-C2-2b</th>
<th>20-2b-2b</th>
<th>734-2b-2b</th>
<th>hL243γ4p</th>
<th>v-mab + hL243γ4p + 734-2b-2b</th>
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<tr>
<td>NHL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daudi</td>
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<td>125</td>
<td>95</td>
<td>4 × 10^-4</td>
<td>25</td>
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<tr>
<td>Raji</td>
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<td>118</td>
<td>70</td>
<td>15.56</td>
<td>2</td>
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<tr>
<td>Ramos</td>
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<td>&gt;40</td>
<td>82</td>
<td>31.04</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Jeko-1</td>
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<td>&gt;200</td>
<td>90</td>
<td>4.40</td>
<td>&gt;2.0</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAG</td>
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<td>55</td>
<td>95</td>
<td>0.53</td>
<td>1</td>
</tr>
<tr>
<td>NCI-H929</td>
<td>0.61</td>
<td>1</td>
<td>98</td>
<td>0.56</td>
<td>1</td>
</tr>
<tr>
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<td>34.12</td>
<td>&gt;2</td>
<td>52</td>
<td>ND</td>
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<tr>
<td>KMS12-PE</td>
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<td>10</td>
<td>83</td>
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<td>&gt;500</td>
<td>99</td>
<td>31.28</td>
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<tr>
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<td>92</td>
<td>0.09</td>
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</table>

Abbreviations: I-EC50, total IFNγ concentration (nmol/L) resulting in 50% growth inhibition compared with untreated cells; ND, treatment failed to reach 50% inhibition; TI, fold reduction in EC50 compared with nontargeted IFNγ (734-2b-2b); lmax, maximal percentage of decrease in viable cells compared with untreated cells.

EC50 for hL243γ4p IgG.

v-mab excluded.
20-C2-2b (I-EC50 = 10 pmol/L) exhibited considerably enhanced potency (TI = 55) for CAG. Apoptosis of CAG was evident following treatment with hL243 + 734-2b at 1 nmol/L, but not at 0.1 or 0.01 nmol/L (Fig. 3B). 20-C2-2b induced apoptosis even at 0.01 nmol/L, and the level observed for 0.1 nmol/L 20-C2-2b was equal or higher than that resulting from any other treatment at 10-fold higher (1 nmol/L) concentration.

Enhanced potency of 20-C2-2b was evident, but lower, for OPM6 (TI = 2), U266 (TI = 7), and MM.1R (TI = 10), which were each highly IFNα-responsive but had lower HLA-DR density and were not inhibited by hL243γ4p. No increased potency was observed for 20-C2-2b on NCI-H929, which was highly IFNα-responsive but is HLA-DR−.

KMS12-BM has high HLA-DR and CD20 antigen densities, and surprisingly, was inhibited by 20-2b-2b (I-EC50 = 31 nmol/L) but not 734-2b-2b (I-EC50 >100 nmol/L) or v-mab. KMS12-BM was more responsive to v-mab + hL243 + 734-2b (EC50 = 3 nmol/L) compared with hL243 + 734-2b (EC50 = 0.7 nmol/L), which in turn was superior to hL243γ4p alone (EC50 = 3.5 nmol/L). Each of these treatments resulted in a strong induction of apoptosis, with the relative levels consistent

Figure 3. Apoptosis in NHL and MM cells. Cells were treated for 48 h before quantification of the percentage of Annexin V–positive cells by flow cytometry. A, for Daudi: v-mab and hL243γ4p were 10 pmol/L; 20-C2-2b, 20-2b-2b, and V + L243 + 2b (a mixture of v-mab, hL243γ4p, and 734-2b-2b) were 1 pmol/L. For Jeko-1, all treatments were at 0.5 nmol/L. B, CAG was treated at 1, 0.1, and 0.01 nmol/L. C, KMS12-BM was treated at 20 and 2 nmol/L. V + L243, a mixture of v-mab and hL243γ4p; L243 + 2b, a mixture of hL243γ4p and 734-2b-2b.

Figure 4. Characterization of MM cell lines. A, antigen densities of HLA-DR and CD20 on selected myeloma lines. After 30 min of incubation with hL243γ4p, v-mab, or hMN-14 (isotype control MAb), cells were probed with PE goat anti-human IgG (Fab) and analyzed by flow cytometry. B, relative sensitivity of myeloma lines to IFNα2. Cells were incubated in the presence or absence of 3 nmol/L of peginterferon alfa-2b for 4 d prior to quantification of viable cells with MTS.
with the *in vitro* cytotoxicity results (Fig. 3C). Additionally, v-mab + hL243 induced more apoptosis than hL243γ4p alone, but less than v-mab + hL243 + 734-2b. These results suggest that for the HLA-DR+/CD20+ MM cells, the activity of all three components of 20-C2-2b (EC50 = 0.1 nmol/L) could contribute to cytotoxicity when combined, even though two of them have virtually no effect when used alone. Evaluation of two additional DNL constructs in KMS12-BM helped elucidate the enhanced potency of 20-C2-2b. A MAb-IFNα designated C2-2b-2b, which comprises hL243 IgG1 and tetrameric IFNα2b (twice that of 20-C2-2b), exhibited less potent cytotoxicity (EC50 = 0.4 nmol/L) and weaker apoptosis induction compared with 20-C2-2b, supporting a contribution of v-mab. More revealing was the finding that 20-C2-C2, a bispecific MAb comprising v-mab and four HLA-DR Fabs, showed high-level induction of apoptosis and >50-fold enhanced potency (EC50 = 0.06 nmol/L) compared with hL243γ4p, indicating that crosslinking of HLA-DR and CD20, which occurs with 20-C2-2b, effectively induces cytotoxicity, perhaps via a unique signaling cascade. Although each construct was potent (EC50<0.5 nmol/L), 20-C2-2b (Iₘₐₓ = 67%) and C2-2b-2b (Iₘₐₓ = 70%) did not kill KMS12-BM as effectively as 20-C2-2b (Iₘₐₓ = 99%), supporting the requirement of all three components for achieving the maximal effect. That the v-mab + hL243 + 734-2b (Iₘₐₓ = 87%) mixture was the only other treatment resulting in >70% killing substantiates this hypothesis.

Together, these data show that antigen density and sensitivity to the actions of IFNα2b, as well as those of the targeting MAbs, are all important determinants of the *in vitro* responsiveness of a particular cell line to the various MAb-IFNα. However, *in vivo* tumor killing may be augmented by ADCC and the actions of immune effector cells, which can be stimulated by the high local concentration of IFNα2b.

**Effector functions and stability in human serum**

We previously reported that 20-2b-2b exhibited enhanced ADCC compared with its parent v-mab (10). By design, hL243γ4p has diminished ADCC (11); however, 20-C2-2b induced significantly (P = 0.0091) greater ADCC compared with v-mab (Supplementary Fig. S5A). Notably, 20-C2-2b induced...
significantly greater ADCC than either 20-2b-2b ($P = 0.0040$) or 20-C2-2b ($P = 0.0115$), indicating an enhancement of the effector function by the presence of IFNo2b. As shown previously for 20-2b-2b (10), 20-C2-2b does not induce CDC in vitro (Supplementary Fig. S5B).

Two different assays for stability of 20-C2-2b in human serum gave very similar results, indicating a loss of ~3.5% per day with roughly 65% remaining after 11 days at 37°C (Supplementary Fig. S5C).

**Ex vivo depletion of NHL from whole human blood**

As shown in Fig. 6, Daudi cells were depleted from whole blood (ex vivo) more effectively by 20-C2-2b (91%) compared with 20-2b-2b (69%), v-mab (49%), hL243γ4p (46%), or 734-2b-2b (10%). Both targeted MAb-IFNα were less toxic to normal B cells compared with Daudi. Under these conditions, B cells were significantly depleted by 20-C2-2b (57%) and hL243γ4p (41%), but not by v-mab, 734-2b, or 20-2b-2b. Monocytes were depleted by hL243γ4p (48%), 734-2b-2b (30%), and 20-2b-2b (21%), but not by v-mab. 20-C2-2b (98%) was highly toxic to monocytes. None of the agents had a significant effect on T cells. Statistical significance with $P < 0.001$ was determined by Student’s $t$ test for each of the differences in percentage of depletion indicated above.

**Discussion**

We and others have reported that fusion proteins comprising CD20-targeting MAbs and IFNα are more effective against NHL compared with combinations of MAb and IFNα in xenograft and syngeneic mouse models, indicating that MAb-IFNα could overcome the toxicity and pharmacokinetic limitations associated with IFNα (10, 16). Although CD20 is an attractive candidate for targeted MAb-IFNα therapy of B-cell lymphoma, its expression is largely limited to malignancies of this lineage, with some individuals exhibiting low antigen density. Here, we report the first bispecific immunocytokine, 20-C2-2b, which specifically targets IFNo2b to both CD20 and HLA-DR, thus potentially expanding the hematopoietic tumor types amenable to this immunocytokine therapy.

Anti–HLA-DR MAbs efficiently induce apoptosis, which is mediated by direct signaling without the requirement of additional crosslinking, and are also potent inducers of ADCC and CDC (11, 15, 17). Where ADCC may enhance therapeutic potential, CDC is largely responsible for the pathogenesis of side effects associated with the MAb infusion (18). The humanized anti–HLA-DR MAb, hl243γ4p, used as a control in this study was engineered for improved clinical safety by using the constant region of the human IgG1 isotype, resulting in diminished ADCC and CDC (11, 15). 20-C2-2b is unique among anti–HLA-DR MAbs in that it lacks CDC, similar to hl243γ4p, but has potent (enhanced) ADCC, making this agent an attractive candidate for immunotherapy.

In the ex vivo setting, v-mab can deplete cells via signaling-induced apoptosis, ADCC, and CDC; MAb-IFNα can employ enhanced ADCC as well as both MAb- and IFNα2b-induced signaling, but not CDC; and hl243γ4p is limited to only direct signaling (11). Although the full spectrum of IFNα-mediated activation of innate and adaptive immunity that might occur in vivo is not realized in this setting, it provides pharmacodynamic data. 20-C2-2b depleted lymphoma cells

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**Figure 6.** Enhanced depletion of NHL cells from whole blood. Fresh heparinized human blood was mixed with Daudi and incubated with 1 nmol/L of the indicated Mab-IFNα or MAb for 2 d. The effect on Daudi, B cells, T cells, and monocytes was evaluated by flow cytometry. Error bars, SD.
might be a pharmacodynamic effect associated with anti-
HLA-DR targeting. These data suggest that monocyte depletion
HLA-DR as well as IFN
γ might be transient because the monocyte population
would likely be transient because the monocyte population
should be repopulated from hematopoietic stem cells.

The four NHL and eight MM cell lines we studied encompass
the naturally occurring heterogeneity in expression and
antigen density of HLA-DR and CD20, as well as responsiveness
to the actions of IFNα, hL243, and v-mab, which all effect MAb-IFNα immunotherapy. Six and eight (of 12) cell lines were inhibited (Vmax >30%) to varying degrees by hl243y4p and 734-2b-2b, respectively. 20-C2-2b potently inhibited (EC50 <1 nmol/L) 11 of the 12 cell lines, with an EC50 ≤0.01 nmol/L for five. Even the least affected MM line (KMS11), which was not inhibited by 734-2b-2b, was responsive to 20-C2-2b (EC50 = 17 nmol/L). An enhancement in the potency of 20-C2-2b over 734-2b-2b was observed in all of the lines besides NCI-H929, which is HLA-DR−/CD20−.

Higher levels of HLA-DR antigen density as well as responsiveness to hl243 correlated with a greater TI for 20-C2-2b, as well as the significance of targeting, even in vitro. 20-C2-2b was superior to the mixture of v-mab + hl243 + 734-2b in 10 of the lines, further highlighting the effect of tumor targeting, which will be considerably greater in vivo, as shown previously for 20-2b-2b (10). Furthermore, in vivo–targeted MAb-IFNα might elicit a potent antitumor immune response.

Whereas the vast majority of cells comprising primary MM specimens are nonclonogenic and have a plasma cell phenotype (CD138+/CD19−/CD20+), putative MM cancer stem cells are CD138+ and express B-cell surface antigens, including CD45, CD19, CD20, and CD22, reminiscent of memory B cells (19). Although a variety of clinical approaches have produced responses, MM remains largely incurable due to relapses thought to be mediated by cancer stem cells, which are resistant to various therapies (20–23). The B-cell phenotype of the putative stem cells prompted clinical investigation with rituximab in MM; however, limited effects on outcome were realized (24). The in vitro results with KMS12-BM are compelling, because it is CD20+, similar to the proposed MM stem cells. 20-C2-2b exhibited potent cytotoxicity and robustly induced the apoptosis of KMS12-BM. Even though non-targeted MAb-IFNα and v-mab were ineffective as single agents, they both apparently contribute to cytotoxicity when used in combination with hl243. The results also indicate that bispicific binding of CD20 and HLA-DR may induce an additional (potent) signal that further enhances toxicity to these cells and may sensitize them to IFNα.

MAb-IFNα produced by DNL exhibits comparable activity to recombinant IFNα. Recently, Xuan and colleagues reported that anti–CD20-IFNα fusion proteins made by traditional recombinant engineering showed a 300-fold reduction in IFNα activity (16). This is noteworthy in comparisons of similar Daudi xenograft studies, where a single 17-ng dose of 20-2b-2b significantly improved survival (10), compared with three 30-μg doses (>5,000-fold more) used for recombinant anti-CD20-hIFNα (16). Studies using IFNα-secreting tumors showed enhanced immune responses elicited by a localized concentration of IFNα (25). Where this might also be achieved with highly active MAb-IFNα, the reduced activity of traditional recombinant MAb-IFNα might not efficiently recruit and stimulate an antitumor immune response, as was reported by Xuan and colleagues (16).

The bispacific MAb-IFNα 20-C2-2b is attractive for the treatment of NHL because each of the three components is active against this disease. This study suggests that 20-C2-2b may also be useful for the therapy of MM and possibly other hematopoietic malignancies. The prospective uses of 20-C2-2b and other constructs introduced herein (C2-2b-2b and 20-C2-C2) in additional diseases, including various leukemias, are the subject of future studies.

Disclosure of Potential Conflicts of Interest

All authors (except R. Stein) declare financial interests (stock, options, and employment) in Immunomedics, Inc. and IBC Pharmaceuticals, Inc.

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