IFN-γ Enhances the Antimyeloma Activity of the Fully Human Anti–Human Leukocyte Antigen-DR Monoclonal Antibody 1D09C3

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Abstract

To investigate the therapeutic activity of the fully human anti–HLA-DR antibody 1D09C3 in multiple myeloma (MM), we reevaluated HLA-DR expression on CD138$^+$ cells, analyzed the capacity of IFN-γ to up-regulate HLA-DR expression on MM cell lines, and tested the *in vitro* and *in vivo* activity of 1D09C3 alone or in combination with IFN-γ. CD138$^+$HLA-DR$^+$ cells were detected in 31 of 60 patients, with 15 of 60 patients having ≥20% CD138$^+$HLA-DR$^+$ cells (median, 50%; range, 23–100). Because primary plasma cells cannot be efficiently cultured *in vitro*, we used a panel of MM cell lines with a dim/negative to bright HLA-DR expression to evaluate 1D09C3-induced cell death. Annexin V/propidium iodide (PI) staining showed that 1D09C3-induced cell death correlated with constitutive HLA-DR expression. Induction of HLA-DR by IFN-γ restored the sensitivity of HLA-DR dim cell lines to 1D09C3. *In vivo*, the combined IFN-γ/1D09C3 treatment significantly increased the median survival of nonobese diabetic/severe combined immunodeficient mice xenografted with KMS-11 cell line, compared with controls (147 versus 48 days, $P \leq 0.0001$) or mice receiving 1D09C3 alone (147 versus 92 days, $P \leq 0.03$). The better therapeutic activity of IFN-γ/1D09C3 treatment over 1D09C3 alone was further shown by a 2-fold increase of mice being disease-free at 150 days after xenograft (47% versus 25%). No mice experienced any apparent treatment-related toxicity. Our data show that (*a*) one fourth of MM patients express HLA-DR on CD138$^+$ cells and (*b*) IFN-γ–induced up-regulation of HLA-DR results in a potent enhancement of the *in vivo* antimyeloma activity of 1D09C3. [Cancer Res 2007;67(7):3269–75]

Introduction

Multiple myeloma (MM) is a neoplasia of plasma cells hallmarked by tumor cell tropism for the bone marrow and production of monoclonal immunoglobulin detectable in serum and/or urine. The median overall survival for MM patients is 4 to 5 years, ranging from <6 months to >10 years according to distinct prognostic factors (1). Although high-dose melphalan followed by autologous stem cell transplantation represented a significant progress in the treatment of MM and is now considered standard therapy for younger patients, the disease remains incurable in the majority of cases (2, 3).

New agents, such as the immunomodulatory drugs thalidomide and lenalidomide, and the proteasome inhibitor bortezomib, have shown a significant role in patients with relapsed and/or refractory disease as well as in the upfront setting (4–10). Despite these important therapeutic advances, in which survival advantage has been shown compared with standard therapy (6, 10, 11), disease relapse still occurs, and thus new strategies to improve disease control are required (3, 12). Treatments targeting both tumor cells and microenvironment have been highly successful but strategies further targeting tumor cells directly and enhancing host immunity against tumor are key next steps in the continued efforts to combat this disease (12).

The development and approval of monoclonal antibodies (mAb) targeting tumor-specific antigens represents a major advance in the therapy of a variety of neoplastic diseases (13), including non–Hodgkin's lymphomas (14–18) and breast cancer (19, 20). Although the phenotype of MM cells is an important component of tumor heterogeneity and might represent a specific target for new treatments, mAbs targeting malignant myeloma cells have not yet been included as part of myeloma therapy (21–24).

The human leukocyte antigen (HLA)-DR is one of the three highly polymorphic genes of the class II MHC, which, under normal conditions, are selectively expressed on immune cells, including B lymphocytes, activated T lymphocytes, monocytes, and dendritic cells (25). Preclinical evidences suggest that both B-cell tumors (26, 27) and T-cell tumors (28) can be killed *in vitro* and *in vivo* by signals delivered through receptors involved in cell activation and growth, such as HLA-DR (29–31). Constitutive HLA-DR expression on normal B cells is down-regulated during differentiation toward plasma cells (32) due to silencing of class II transactivator (33), which is also involved in suppressing HLA-DR expression on malignant plasma cells (34). Expression of both class II transactivator and HLA-DR in myeloma cells can be up-regulated by IFN-γ, thus representing an interesting area of investigation for specifically targeting malignant plasma cells (35–37).

Recently, an anti–HLA-DR antibody termed 1D09C3 has been generated by screening the human combinatorial antibody library (38). *In vitro*, 1D09C3 exerts a potent tumoricidal activity on several lymphoma and leukemia cell lines, as well as primary cells from chronic lymphocytic leukemia (CLL) patients (38, 39). Cell death induced by 1D09C3 involves a cascade of events, including reactive oxygen species increase, c-Jun-NH_2-kinase activation, and mitochondrial membrane depolarization (39). *In vivo*, injection of

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1D09C3 in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice xenografted with a variety of human malignant lymphoma cell lines results in significant delay of tumor growth and long-term disease-free survival in significant proportions of lymphoma-bearing mice (38, 39). 1D09C3 is currently being tested in two phase I clinical trials in patients with relapsed and refractory B-cell malignancies, including non–Hodgkin's lymphoma, CLL, and Hodgkin lymphoma.

To evaluate in a preclinical setting the therapeutic potential of 1D09C3 in MM patients, we analyzed HLA-DR expression on bone marrow malignant plasma cells enriched from 60 consecutive MM patients, investigated the capacity of IFN-γ to up-regulate HLA-DR expression on myeloma cell lines, and tested the activity of 1D09C3 alone or in combination with IFN-γ in inducing cell death both *in vitro* and *in vivo* using a xenotransplant model of MM in NOD/SCID mice.

Materials and Methods

Reagents. Recombinant human IFN-γ (Imukin) was purchased from Boehringer Ingelheim GmbH (Ingelheim, Germany); 1D09C3 was kindly provided by GPC Biotech (Munich, Germany); and the human IgG4 isotype control (I-4764) was purchased from Sigma-Aldrich (Milan, Italy).

Patients. Between March 2003 and May 2006, 60 consecutive patients with a diagnosis of MM (n = 58) or monoclonal gammopathy of undetermined significance (n = 2) were studied. Patients (females 29, males 31) ranged in age from 28 to 80 years (median, 59 years). The majority of patients (64%) had a stage IIIA or IIIB MM. Mean serum level of β_2-microglobulin was 3.9 mg/L (median, 2.8; range, 0.7–20). Cytogenetic abnormalities were detected in 29 of 58 analyzed patients, with 12 patients showing a 13q14 deletion, 13 patients a chromosome 13 monosomy, and 4 patients a t(11;14) translocation, whereas in 29 cases no karyotype abnormality could be detected. At the time of analysis, 46 patients were newly diagnosed, 2 were relapsed after allogeneic stem cell transplantation, 1 was relapsed after autologous stem cell transplantation, 8 had been treated with regimens including high-dose dexamethasone [dexamethasone (n = 5); dexamethasone and thalidomide (n = 3)], and 3 had been treated with melphalan and prednisone. The study was approved by institutional ethical committee, and written informed consent was obtained from each patient.

Highly purified plasma cells and cell lines. $CD138^+$ plasma cells were enriched using an immunomagnetic method according to manufacturer's instructions and the AutoMACS device (Miltenyi Biotec, Bergisch-Gladbach, Germany). Enrichment of $CD138^+$ cells resulted in a median purity of 91% (range, 27–100%) and a median recovery of 55% (range, 27–95%). Cell lines included KMS-11, RPMI 8226, OPM-2, U-266, LP-1, and ARH-77, and were purchased from the DSMZ (Braunschweig, Germany).

Flow cytometry. The following mAbs were used: anti–CD138-FITC (clone B-B4) from Serotec (Kidlington, Oxford, United Kingdom), anti–CD45-peridinin chlorophyll protein (clone 2D1), and anti–HLA-DR-phycoerythrin (clone L243), all from Becton Dickinson (San Jose, CA). Highly purified $CD138^+$ cells were analyzed for HLA-DR expression by three-color flow cytometry (CD138/HLA-DR/CD45) using a FACSCalibur flow cytometry system (Becton Dickinson) equipped with a Macintosh PowerMac G4 personal computer (Apple Computer, Inc., Cupertino, CA) and Cell Quest (Becton Dickinson) software. Briefly, CD138-enriched cells (0.5×10^6) were incubated with the appropriate antibody combination or isotype controls. The expression of HLA-DR on $CD138^+$ cells was measured as percentage of positive cells according to gates set using the isotype control staining. Bone marrow samples were defined positive for HLA-DR when ≥20% $CD138^+$ cells were $HLA\text{-}DR^+$. The intensity of HLA-DR expression on $CD138^+$ plasma cells was measured in terms of mean fluorescence intensity ratio (MFI-R), which was calculated as MFI of anti–HLA-DR stained samples/MFI of isotype control stained samples.

Viable cell countings. MM cell lines (0.5×10^5/mL to 1×10^5/mL) resuspended in culture medium supplemented with heat-inactivated serum were exposed to IFN-γ (96 h, 100 IU/mL), 1D09C3 (24 h, 2.5 μg/mL), or IFN-γ plus 1D09C3. Controls were untreated or were exposed to the murine anti–HLA-DR 10F12 antibody that fails to induce cell death (40). At the end of incubation, viable and dead cells were distinguished by PI staining and fluorescence-activated cell sorting (FACS) analysis. To obtain absolute cell counts by FACS, cell samples were supplemented with Flowcount beads. Cell counts were calculated by the following equation: viable cells × total beads/counted beads.

Annexin V/PI staining. The Annexin V-FITC assay (Bender MedSystems, San Bruno, CA) was used to quantitatively determine the percentage of cells undergoing apoptosis after exposure to IFN-γ and/or 1D09C3 (41, 42). Controls were either without antibody or with the murine anti–HLA-DR 10F12 antibody (40). Briefly, cells to be analyzed were washed twice with cold PBS and then resuspended in binding buffer [10 nmol/L HEPES, 140 nmol/L NaCl, 5 nmol/L $CaCl_2$ (pH 7.4)]. Following incubation, 0.1 mL of the cell suspension was transferred to a 5 mL culture tube, and 5 μL of Annexin V-FITC were added. After vortexing, samples were incubated for 10 min at room temperature in the dark. At the end of the incubation, 0.2 mL of binding buffer and 10 μL of PI were added, and the cells were analyzed immediately by flow cytometry. The Annexin V/PI double staining allowed to distinguish between apoptotic (Annexin V+/PI−) and nonapoptotic [i.e., dead cells (Annexin V+/PI+ plus Annexin V−/PI+)].

***In vivo* tumoricidal activity in NOD/SCID mice.** Six- to eight-week-old female NOD/SCID mice with body weight of 20 to 25 g were purchased from Charles River (Milan, Italy). Mice were housed under standard laboratory conditions according to our institutional guidelines. Animal experiments were done according to the Italian laws (D.L. 116/92 and following additions), which enforce the EU 86/109 Directive and were approved by the institutional Ethical Committee for Animal Experimentation. On day 0, mice were given cyclophosphamide i.p. (50 mg/kg), and on day 1 were inoculated with KMS-11 cells i.v. (0.5×10^6 per mouse). Mice were checked twice weekly for body weight measurements and toxicity. End point of *in vivo* experiments was death. Treatment groups included mice receiving IFN-γ (10^4 IU/mouse; i.p.; days 1, 2, 3, 6, 7, 8, and 9), 1D09C3 (1 or 2 mg/mouse; s.c.; days 3, 7, 9), or IFN-γ plus 1D09C3 (1 mg/mouse; s.c.; days 3, 7, and 9). Control mice received PBS or the human IgG4 isotype control (1 mg/mouse; s.c.; days 3, 7, and 9) as an irrelevant antibody control. Experiments were done on four separate occasions, using five mice per treatment group.

Histologic analysis and immunohistochemistry. Mice bearing s.c. tumor nodules (~ 10 mm in diameter) of KMS-11 cells (5×10^6 per mouse) were treated with IFN-γ and after 48 h tumors were excised and analyzed. Formalin-fixed, paraffin-embedded tumor nodules were dewaxed, hydrated, sectioned at 3 μm, and stained with H&E or processed for immunohistochemistry by using a mouse anti–human HLA-DR mAb (clone LN3, Alexis Corporation, Lausen, Switzerland). Immunostaining was done using an

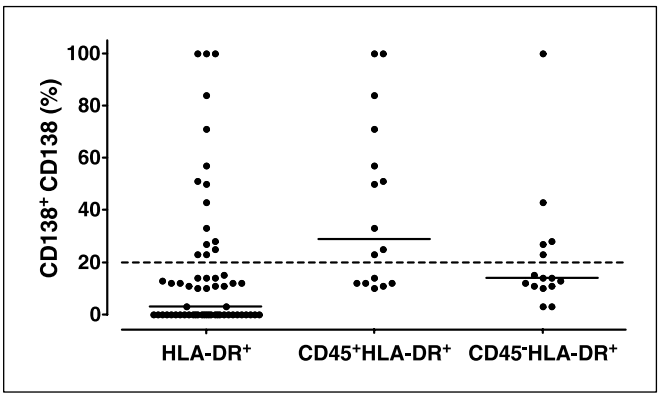

Figure 1. HLA-DR expression on primary cells. Expression of HLA-DR on $CD138^+$ cells measured by flow cytometry as percentage of positive cells according to gates set using the isotype control staining. $CD138^+$ cells were enriched from the bone marrow of MM patients (n = 60). For patients with $CD138^+HLA\text{-}DR^+$ cells (n = 31), HLA-DR expression is also shown according to CD45 expression. *Black lines,* median values. *Dotted line,* 20% cutoff level.

Table 1. HLA-DR expression on $CD138^+$ plasma cells

	Patients with ≥1% HLA-DR$^+$ plasma cells	% HLA-DR$^+$ plasma cells in patients with ≥1% HLA-DR$^+$ plasma cells	Patients with ≥20% HLA-DR$^+$ plasma cells	% HLA-DR$^+$ plasma cells in patients with ≥20% HLA-DR$^+$ plasma cells
$CD138^+$ cells	52% (31/60)*	3 (17; 0–100)†	25% (15/60)*	50 (54; 23–100)†
$CD138^+CD45^+$ cells	52% (16/31)	29 (42; 10–100)	67% (10/15)	54 (59; 23–100)
$CD138^+CD45^-$ cells	48% (15/31)	14 (22; 3–100)	33% (5/15)	28 (44; 23–100)

*(Absolute numbers).
†Median (mean; range).

automated immunostainer (Nexes, Ventana Medical Systems, Inc., Tucson, AZ) according to a modified version of the company protocols.

Statistical analysis. Statistical analysis was done with the statistical package Prism 4.0 (GraphPad Software, San Diego, CA) run on a Macintosh G4 personal computer (Apple Computer). To determine whether there was a nonrandom association between two categorical variables, Fisher's exact test was used. To test the probability of significant differences between untreated and treated samples, the Student's t test for paired data (two-tail) was used. Survival curves were created using the product limit method of Kaplan-Meier, and survival differences were compared using the log-rank test. Differences were considered significant if $P \leq 0.05$.

Results

HLA-DR expression on primary $CD138^+$ cells. Analysis of HLA-DR expression on $CD138^+$ cells from 60 bone marrow samples revealed a wide degree of heterogeneity in terms of fluorescence intensity [median MFI-R of 4.3 (mean, 21); range, 2.4–89] as well as percentages of $CD138^+$HLA-DR^+ cells. Overall, $CD138^+$HLA-DR^+ cells were detected in 31 of 60 patients (52%; Fig. 1), with the median percentage of $CD138^+$HLA-DR^+ cells being 3% (mean, 17%; range, 0–100%; Table 1). Fifteen of 60 patients (25%) had ≥20% $CD138^+$HLA-DR^+ plasma cells, with three patients (5%) expressing HLA-DR on 100% of their tumor cells (Fig. 1). In HLA-DR^+ patients (i.e., those with ≥20% $CD138^+$HLA-DR^+ marrow plasma cells), the median percentage of $CD138^+$HLA-DR^+ plasma cells was 50% (mean, 54%; range, 23–100; Table 1). HLA-DR expression on $CD138^+$ cells was not associated with adverse prognostic factors, including disease stage, 13q14 deletion or chromosome 13 monosomy, or serum levels of β_2-microglobulin. Because $CD45^+$ plasma cells are likely to be important in the pathogenesis and progression of MM, HLA-DR expression was analyzed on $CD138^+CD45^+$ and $CD138^+CD45^-$ plasma cells. The median percentage of $CD138^+$ plasma cells that expressed CD45 was 6% (mean, 35%; range, 0–100). Overall, the median percentages of $CD138^+$HLA-DR^+ expressing or lacking CD45 were 29% and 14%, respectively (Table 1). Among HLA-DR^+ patients, 10 (67%) and 5 (33%) of 15 patients were $CD138^+CD45^+$HLA-DR^+ and $CD138^+CD45^-$HLA-DR^+, respectively (Fig. 1).

HLA-DR expression on MM cell lines. Due to the inherent difficulties in culturing primary plasma cells, a panel of MM cell lines with heterogeneous HLA-DR expression was selected to investigate whether constitutive as well as IFN-γ–induced HLA-DR could be targeted by 1D09C3. As shown in Fig. 2*A*, LP-1, ARH-77, and U-266 were 100% HLA-DR positive with a bright HLA-DR expression, as shown by MFI-Rs of 63, 110, and 315, respectively (Fig. 2*B*). Constitutive HLA-DR expression detected in these cell lines was further increased upon culture with IFN-γ. In contrast, OPM-2, RPMI 8226, and KMS-11 showed 0%, 7%, and 55% HLA-DR^+ cells, respectively, with negative or dim antigen expression (Fig. 2*B*). Upon exposure to IFN-γ, RPMI 8226 and KMS-11, but not OPM-2 cell lines, showed a statistically significant increase of HLA-DR

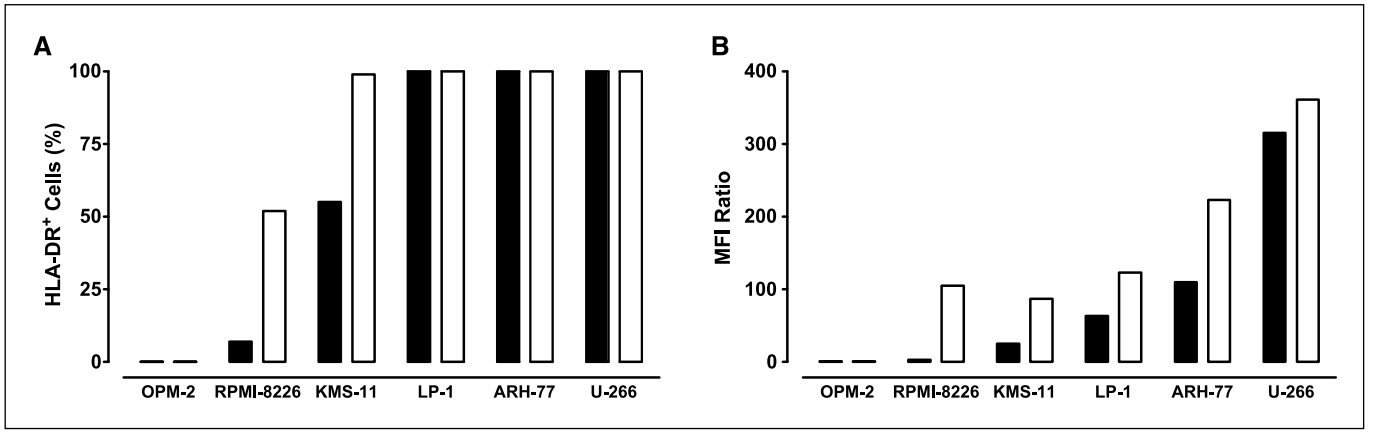

Figure 2. HLA-DR expression on MM cell lines. *A,* percentages of HLA-DR^+ cells on MM cell lines before (*filled columns*) and after (*open columns*) exposure to IFN-γ. *B,* fluorescence intensity of HLA-DR before (*filled columns*) and after (*open columns*) exposure to IFN-γ. Intensity of antigen expression was measured as MFI-R, which was calculated as MFI of anti–HLA-DR stained samples/MFI of isotype control stained samples. Each cell line was tested in three independent experiments. *Columns,* values from one representative experiment. ■, control; □, IFN-γ.

expression both in terms of percentage of positive cells and intensity of antigen expression (Fig. 2*A–B*).

***In vitro* antiproliferative activity of 1D09C3 and/or IFN-γ.** The effect of 1D09C3 on the *in vitro* growth of untreated as well as IFN-γ–treated cell lines was then investigated. Treatment of RPMI 8226 and OPM-2 with 1D09C3 alone failed to affect both viable cell countings and cell death, and resulted in an average 25% reduction of viability of KMS-11 cell line (i.e., the cell line with a dim HLA-DR expression; Fig. 3*A* and *B*). In contrast, exposure of HLA-DR$^+$ cell lines to 1D09C3 significantly reduced mean viable cell countings (100% versus 24%, $P \leq 0.001$) and increased mean cell death (20% versus 44%, $P \leq 0.001$), compared with controls (Fig. 3*C* and *D*). IFN-γ used as single agent reduced, although at a variable degree, viable cell countings in four of six cell lines (Fig. 3*A–C*).

As compared with controls, exposure of RPMI 8226 and KMS-11 cell lines to the combined IFN-γ/1D09C3 treatment significantly reduced viable cell countings to 50% ($P \leq 0.0001$) and 29% ($P \leq 0.0001$), respectively, and increased cell death to 45% ($P \leq 0.0001$) and 40% ($P \leq 0.0001$), respectively (Fig. 3*A–B*). In contrast, the combined treatment was ineffective on OPM-2 cell line, which failed to show any HLA-DR induction upon pretreatment with IFN-γ, as well as three of three HLA-DR$^+$ cell lines (Fig. 3).

***In vivo* tumoricidal activity.** To determine whether the combined IFN-γ/1D09C3 treatment could enhance the antimyeloma activity not only *in vitro* but also *in vivo*, a xenograft model of MM was used. KMS-11 cell line was selected for *in vivo* studies due to its HLA-DR expression, which mimics antigen expression usually detected on primary CD138$^+$ plasma cells. KMS-11 xenograft is characterized by a progressive bone marrow infiltration detectable by day 14 after tumor inoculation when tumor cells represent on average 25% to 30% of marrow cellularity. Skeletal lesions in the skull as well as vertebrae and extraskeletal lesions, including plasmacytomas in soft tissues, can be detected in >50% of xenografted mice. The development of spinal lesions is associated with hind-leg paralysis (95% of mice), usually occurring 5 to 6 weeks after xenografting. Additionally, a monoclonal κ light chain of human type can be detected in the serum of KMS-11–bearing NOD/SCID mice.

Consistent with *in vitro* data, anti–HLA-DR immunostaining staining of sections from tumor nodules growing in mice injected s.c. with KMS-11 cell line revealed that treatment with IFN-γ induced a marked induction of HLA-DR expression both in terms of percentage of positive cells as well as intensity of antigen expression (Fig. 4*A* and *B*). Treatment with PBS or IFN-γ resulted in median survival times of 48 and 50 days, respectively (Fig. 4*C*). Treatment with the human IgG4 isotype control used as an irrelevant antibody resulted in a survival similar to that of PBS-treated mice (data not shown). A significant increase of median

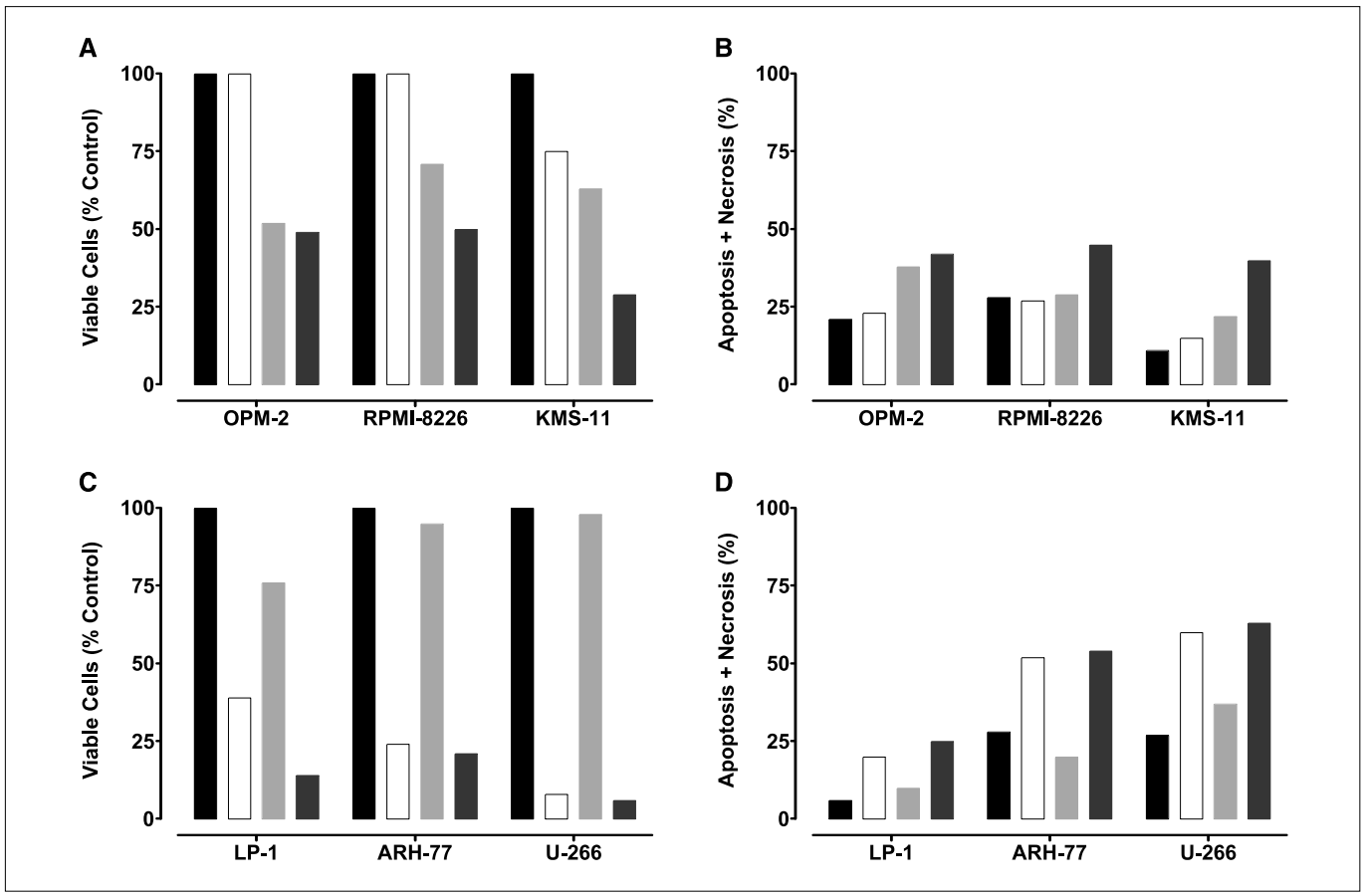

Figure 3. 1D09C3-induced cell death. Viable cells (*A*) and cell death (*B*) of MM cell lines with dim or absent HLA-DR expression upon incubation with 1D09C3 (2.5 μg/mL, 24 h), IFN-γ (100 IU/mL, 48–72 h), or IFN-γ plus 1D09C3. Viable cells (*C*) and cell death (*D*) of HLA-DR$^+$ MM cell lines upon incubation with 1D09C3, IFN-γ, or IFN-γ plus 1D09C3. *A* and *C,* obtained by PI staining and FACS analysis. Absolute cell counts by FACS were obtained by supplementing cell samples with Flowcount beads. *B* and *D,* obtained by flow cytometry using the Annexin V/PI double staining and represent the sum of apoptotic (Annexin V$^+$/PI−) and nonapoptotic (i.e., dead) cells (Annexin V$^+$/PI$^+$ plus Annexin V−/PI$^+$). Each cell line was tested in three independent experiments. *Columns,* values from representative experiment. ■, control; □, 1D09C3; ■, IFN-γ; ■, IFN-γ + 1D09C3.

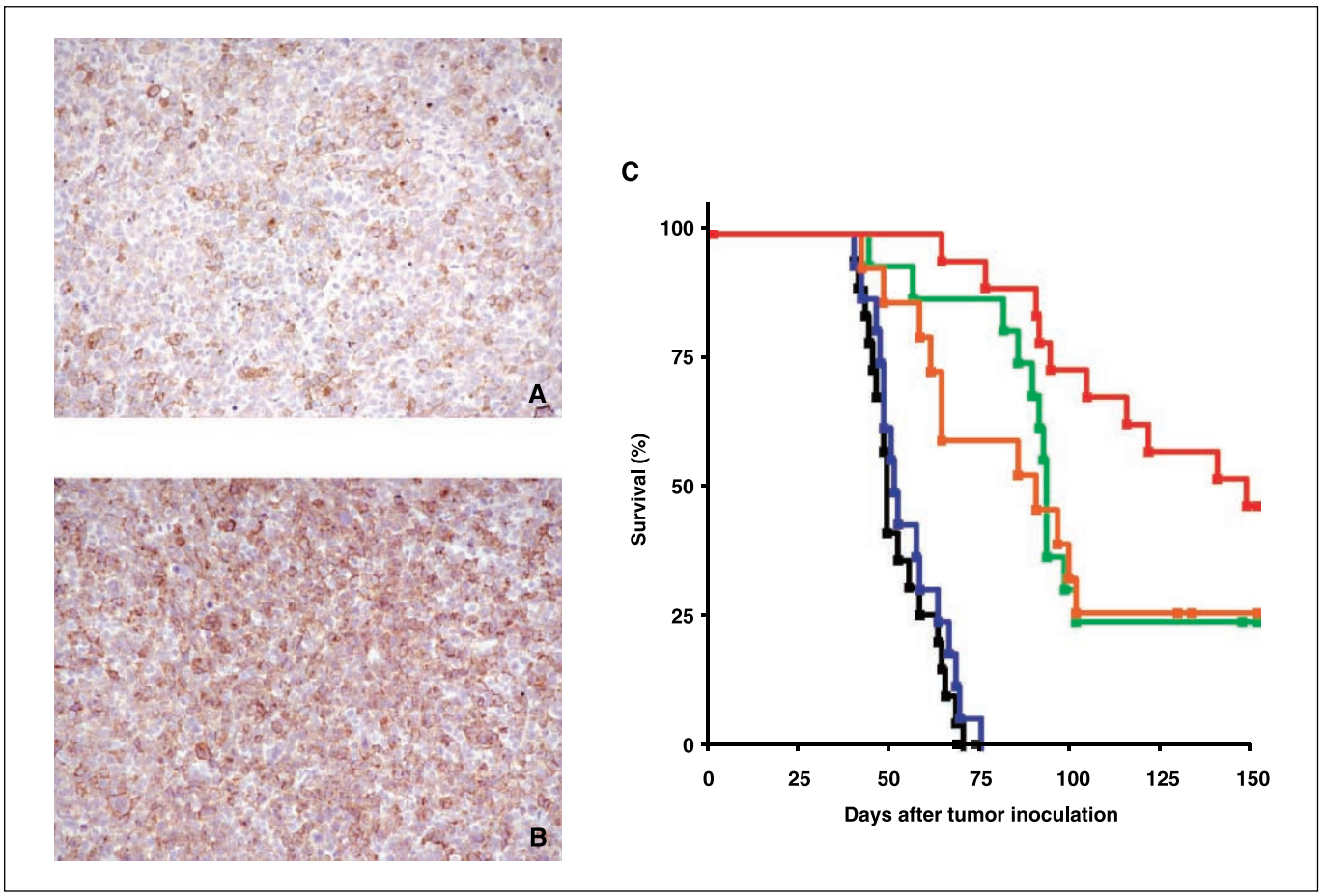

Figure 4. IFN-γ up-regulates HLA-DR expression *in vivo* and enhances the therapeutic activity of 1D09C3. Anti–human HLA-DR staining of sections from tumor nodules growing in mice injected s.c. with KMS-11 cell line. Tumor nodules (~10 mm in diameter) were excised and stained 24 h after injection of PBS buffer control (*A,* magnification ×40) or IFN-γ (10^4 IU/mouse; i.p.; days 1, 2, and 3; *B,* magnification ×40). *C,* Kaplan-Meier estimates of overall survival of NOD/SCID mice xenografted with KMS-11 cell line (0.5×10^6 cells/mouse, i.v.). Mice received PBS (*black line*), IFN-γ (10^4 IU/mouse; i.p.; days 1, 2, 3, 6, 7, 8, and 9; *blue line*), 1D09C3 at 3 mg/mouse (*green line*) or 6 mg/mouse (*yellow line*; s.c.; days 3, 7, and 9), or IFN-γ plus 1D09C3 (3 mg/mouse, *red line*). Survival was measured from the day of xenografting. Each treatment group contained 20 mice.

survival over controls was detected in mice treated with 1D09C3 alone at either 3 mg/mouse (48 versus 92 days, $P \leq 0.0001$) or 6 mg/mouse (48 versus 89 days, $P \leq 0.0001$). For both dose levels, treatment with 1D09C3 alone resulted in 25% of NOD/SCID mice alive and disease-free at the end of the 150-day observation period. The combined treatment with IFN-γ plus 1D09C3 (3 mg/mouse) resulted in a significant increase of median survival compared with controls (48 versus 147 days, $P \leq 0.0001$) as well as mice receiving 1D09C3 alone at 3 mg/mouse (92 versus 147, $P \leq 0.04$) or 6 mg/mouse (89 versus 147, $P \leq 0.03$). The significant enhancement of therapeutic activity of the combined IFN-γ/1D09C3 treatment over 1D09C3 alone was further supported by an increase up to 47% of animals being alive and well at the end of the 150-day observation period. No mice experienced any apparent treatment-related toxicity.

Discussion

A single autologous transplantation is now considered standard therapy for younger MM patients (4). Tandem autotransplantation has significantly increased both overall and event-free survival (43, 44); however, due to age restrictions, its feasibility is limited to a small subset of young MM patients. Moreover, with the advent of novel therapies, its effect remains controversial. Despite the introduction of new drugs that further contributed at improving the outcome of MM (11, 45), virtually all patients ultimately relapse, suggesting that new treatments specifically targeting the neoplastic clone and/or its microenvironment are required (12). The self-renewal capacity of plasma cells in the SCID-human mouse model suggests that these apparently terminally differentiated B cells retain self-renewal capacity, thus critically contributing to disease relapse (46). Additionally, the presence of somatic hypermutations of immunoglobulin variable region genes is consistent with an immortalizing event during plasma cell generation in germinal centers of lymph nodes (47), suggesting that plasma cell phenotype may indeed represent a specific target for new treatments (48).

Class II antigens represent an emerging therapeutic target in lymphoproliferative disorders, due to the development and clinical availability of several anti–HLA-DR mAbs, including the murine Lym-1 (49), the humanized Hu1D10 (apolizumab; ref. 50), and the fully human antibody named 1D09C3 (38). The latter antibody has a strong selectivity toward neoplastic cells, an inherent tumoricidal activity; that is, it does not require intact immunologic effector mechanisms of the patient and exerts potent antitumor effects

in vivo in NOD/SCID mice in several clinical models of either minimal tumor burden or disseminated disease (38, 39).

In view of the availability of 1D09C3 for future phase II studies, we reevaluated HLA-DR expression in MM to find out whether 1D09C3 might represent a treatment modality to target malignant plasma cells. Expression of HLA-DR on plasma cells was detected in 31 of 60 patients (52%). Fifteen of 60 patients (25%) had ≥20% $CD138^+$ plasma cells expressing the HLA-DR antigen, with three patients (5%) expressing HLA-DR on 100% of their tumor cells. These data suggest that targeting HLA-DR antigen would substantially affect the neoplastic clone in at least one fourth of MM patients. Two separate populations of plasma cells have been reported on the basis of CD45 expression (51) and $CD45^+$ plasma cells are likely to be important in the pathogenesis and progression of MM (52). Indeed, two thirds of $HLA\text{-}DR^+$ patients express CD45 on their plasma cells, suggesting that 1D09C3 might represent an appropriate treatment modality to target self-renewing, malignant plasma cells. HLA-DR expression on plasma cells was not associated with specific disease stages or distinct cytogenetic abnormalities.

Because primary plasma cells cannot be efficiently cultured *in vitro* and xenografted *in vivo*, we used a panel of MM cell lines characterized by a heterogenous HLA-DR expression resembling that detected on primary plasma cells to evaluate the potential clinical relevance of HLA-DR targeting. *In vitro*, 1D09C3-induced cell death strongly correlated with constitutive HLA-DR expression. Because expression of both class II transactivator and HLA-DR can be efficiently up-regulated by IFN-γ at least in cell lines with a dim HLA-DR expression, we sought to determine whether IFN-γ–induced HLA-DR expression could restore the sensitivity of 1D09C3-unresponsive cell lines to the cytotoxic activity of the antibody. Indeed, an efficient HLA-DR induction upon culture with IFN-γ was associated with a marked triggering of 1D09C3-induced cell death, whereas failure to induce HLA-DR expression resulted in no sensitization to 1D09C3. Translating these results from cell lines to primary cells would imply that IFN-γ exposure could substantially enhance HLA-DR expression at least in those patients having a dim antigen expression on their plasma cells; that is, the combined IFN-γ/1D09C3 treatment could result in an efficient antimyeloma activity in at least 50% of MM patients.

Immunomodulatory drugs have been shown to up-regulate IFN-γ production and increase both natural killer selectivity as well as affect T-cell subsets (53–55). Moreover, recent data has suggested that immunomodulatory drugs can also favorably influence the activity of dendritic cells in this setting (56). The data supporting the immunomodulatory role of these agents, including thalidomide and lenalidomide, have been derived preclinically and now most recently clinically (10, 57). Therefore, a combination therapy using 1D09C3 in association with immunomodulatory drugs might represent a compelling avenue.

KMS-11 cell line was selected for *in vivo* studies due to the specific features of this cells, including expression of HLA-DR at low intensity, limited responsiveness to 1D09C3 *in vitro*, and consistent tumorigenic activity *in vivo*. Injection of 1D09C3 alone in NOD/SCID mice xenografted with KMS-11 cells induced a significant prolongation of median survival and a 25% incidence of long-term survivors. There was no survival difference between mice receiving 1D09C3 at 3 or 6 mg/mouse, suggesting that the 3 mg dose level was capable of fully targeting all the antigen sites. Interestingly, mice pretreatment with IFN-γ resulted in a marked HLA-DR induction, which translated in a significant enhancement of 1D09C3 antimyeloma activity, as shown by a 3-fold increase of median survival and 50% incidence of long-term survivors in mice receiving IFN-γ plus 1D09C3.

In conclusion, our data show that a substantial proportion of patients with MM express HLA-DR on $CD138^+$ cells and that expression of this antigen can be efficiently up-regulated by IFN-γ, resulting in a marked enhancement of the *in vivo* therapeutic efficacy of 1D09C3. These findings will require further studies and most importantly translation into clinical trials eventually combining 1D09C3 with immunomodulatory drugs or proteasome inhibitors to further improve patient outcome.

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