(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 January 2008 (17.01.2008)

PCT

(10) International Publication Number
WO 2008/008455 A2

(51) International Patent Classification:
A61K 39/395 (2006.01)

(21) International Application Number:
PCT/US2007/015926

(22) International Filing Date: 13 July 2007 (13.07.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/830,679 14 July 2006 (14.07.2006) US

(71) Applicant (*for all designated States except US*): **THE UNIVERSITY OF MIAMI** [US/US]; 1600 N.W. 10th Avenue, Miami, FL 33156 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **LEE, Kelvin, P.** [US/US]; c/o The University Of Miami, 1600 N.W. 10th Avenue, Miami, FL 33156 (US). **BOISE, Lawrence, H.** [US/US]; c/o The University Of Miami, 1600 N.W. 10th Avenue, Miami, FL 33156 (US).

(74) Agent: **WILSON, Mary, J.**; Nixon & Vanderhye P.C., 901 North Glebe Road, 11th Floor, Arlington, VA 22203-1808 (US).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2008/008455 A2

(54) Title: METHOD OF TREATING MULTIPLE MYELOMA

(57) Abstract: The present invention relates, in general to multiple myeloma and, in particular, to methods of treating multiple myeloma by modulating CD28-mediated regulation of multiple myeloma cell proliferation and survival. The invention also relates to compounds and compositions suitable for use in such methods.

METHOD OF TREATING MULTIPLE MYELOMA

This application claims priority from U.S. Provisional Application No. 60/830,679, filed July 14, 2006, the entire content of which is incorporated herein by reference.

5 TECHNICAL FIELD

The present invention relates, in general to multiple myeloma and, in particular, to methods of treating multiple myeloma by modulating CD28-mediated regulation of multiple myeloma cell proliferation and survival. The invention also relates to compounds and compositions suitable for use in such 10 methods.

BACKGROUND

Multiple myeloma (MM) remains an incurable clonal B lymphoid neoplasm of plasma cells, second only to non-Hodgkin's lymphoma in incidence (Anderson et al, Hematology, Am. Soc. Hematol. Educ. Program 63-88 (1998)). 15 Despite significant initial responses to chemotherapy, >90% of patients with MM relapse with resistant disease (Lokhorst et al, Br. J. Haematol. 106:18-27 (1999)), underscoring the need to identify novel therapeutic targets that affect myeloma survival and resistance pathways. Given that MM cells are critically dependent on normal elements of the bone marrow stroma for cell growth and survival, these 20 interactions are attractive targets. One such interaction is stromal production of soluble growth factors, such as IL-6 and TRANCE (Anderson et al, Hematology, Am. Soc. Hematol. Educ. Program 63-88 (1998); Pearse et al, Proc. Natl. Acad. Sci. USA 98:11581-11586 (2001)). Another important set of interactions involves direct myeloma cell contact with extracellular matrix (ECM) and/or

stromal cells. Such direct contact upregulates stromal cell IL-6 and VEGF production, induces NFκB signaling, drops myeloma cells out of cell cycle and enhances resistance to chemotherapy (Damiano et al, Blood 93:1658-1667 (1999); Hazlehurst et al, Oncogene 19:4319-4327 (2000); Landowski et al, Oncogene 22:2417-2421 (2003)). However, the specific molecular (e.g., integrins (Damiano et al, Blood 93:1658-1667 (1999); Sanz-Rodriguez and Teixido, Leuk. Lymphoma 41:239-245 (2001)) and cellular (e.g., osteoclasts (Yaccoby et al, Cancer Res. 64:2016-2023 (2004)) components of these direct interactions within the complex bone marrow microenvironment are only beginning to be described. As important, the characteristic progression of myeloma to stromal-independence marks a clinically worse disease (Tricot et al, Br. J. Haematol. 125:24-30 (2004)), yet the mechanisms that underlie this transition are also poorly understood.

Identification of pro-survival receptors typically expressed on myeloma cells may point to the stromal cells expressing the receptor ligands. One potential receptor is CD28. CD28 has a restricted lineage expression, found predominantly on T cells but also on normal plasma cells, primary myeloma isolates and myeloma cell lines at levels comparable to T cells (Kozbor et al, J. Immunol. 138:4128-4132 (1987); Lee et al, J. Immunol. 145:344-352 (1990); Robillard et al, Clin. Cancer Res. 4:1521-1526 (1998); Kornbluth, Curr. Top. Microbiol. Immunol. 194:43-49 (1995)). In T cells, CD28 receptor activation occurs following binding to its ligands, CD80 (B7-1) and CD86 (B7-2), which are expressed predominantly on professional antigen presenting cells (APC), and in particular dendritic cells (DC) (Sharpe and Freeman, Nat. Rev. Immunol. 2:116-126 (2002)). The signaling pathways downstream of the CD28 receptor in T cells include PI-3 kinase→PDK1→Akt and Vav→Rac1/Cdc42→MEKK (both which regulate NFκB activation) (Rudd and Schneider, Nat. Rev. Immunol. 3:544-556 (2003)). Importantly, in myeloma cells ,PI3K/Akt signaling transduces the anti-apoptotic effects of IL-6 and insulin-like growth factor 1 (IGF-1) (Tu et al, Cancer

Res. 60:6763-6770 (2000)), and for IGF-1 involves sustained activation of NFκB (Mitsiades et al, Oncogene 21:5673-5683 (2002)). Functionally, CD28 delivers the costimulatory signal that, in conjunction with T cell receptor signaling, results in augmented T cell proliferation, effector function (Shahinian et al, Science 5 261:609-612 (1993); Lindstein et al, Science 244:339-343 (1989)) and enhanced survival via upregulation of anti-apoptotic gene bcl-x_L (Boise et al, Immunity 3:87-98 (1995)) and more efficient glucose metabolism (Frauwirth et al, Immunity 16:769-777 (2002)).

In contrast to T cells, little is known about CD28 function in myeloma 10 cells. Clinically, however, CD28 expression highly correlates with myeloma disease progression, such that high CD28 expression is seen in 26% of newly diagnosed myelomas, 59% of medullary relapses, 93% of extramedullary relapses and 100% of secondary plasma cell leukemias (including nearly all the human and murine MM cell lines) (Robillard et al, Clin. Cancer Res. 4:1521-1526 (1998); 15 Shapiro et al, Blood 98:187-193 (2001)). Moreover, myeloma cell expression of CD28 in newly diagnosed patients is a major prognostic predictor of poor clinical outcome following high dose chemotherapy (Almeida et al, Br. J. Haematol. 107:121-131 (1999); Mateo et al, Haematologica 90:3 (2005)). These clinical findings suggest that CD28 confers a survival advantage to MM cells that express 20 the receptor, resulting in their outgrowth under treatment selection pressure. Additionally, the possibility that CD28 is involved in the progression to stroma-independent MM is supported by observations that primary $CD28^+$ myelomas co-express CD86 (10/10 patient samples (Robillard et al, Clin. Cancer Res. 4:1521-1526 (1998)) and that $CD86^+$ myelomas have a significantly poorer prognosis 25 (Pope et al, Blood 96:1274-1279 (2000)). The possibility of autocrine CD28-CD86 activation is supported by some (Kornbluth, Curr. Top. Microbiol. Immunol. 194:43-49 (1995)) but not all (Zhang et al, Leukemia 12:610-618 (1998)), *in vitro* studies. Other functional studies of CD28 activation in MM cell

lines have been equivocal (Kozbor et al, J. Immunol. 138:4128-4132 (1987); Kornbluth, Curr. Top. Microbiol. Immunol. 194:43-49 (1995)). CD28 activation does not induce IL-6 secretion in MM cells (Shapiro et al, Blood 98:187-193 (2001); Zhang et al, Leukemia 12:610-618 (1998)) like it does in T cells (Lorre et al, Clin. Immunol. Immunopathol. 70:81-90 (1994)) but does upregulate expression of the pro-angiogenic chemokine IL-8 (Shapiro et al, Blood 98:187-193 (2001)). Direct evidence of a pro-survival role for CD28 in myeloma cells has not been reported, although such a role in normal plasma cells is indirectly suggested by previous observations that CD28 knockout mice have markedly diminished serum immunoglobulin levels (Shahinian et al, Science 261:609-612 (1993); Horspool et al, J. Immunol. 160:2706-2714 (1998)), including T-independent antibody responses (Gray et al, J. Immunol. 169:2292-2302 (2002)). Conversely, a recently developed anti-CD28 mAb inhibits MM cell line proliferation and induces some morphological aspects of apoptosis - whether this is due to an activating or blocking effect of the antibody was not determined (Qiu et al, Cell Immunol. 236(1-2):154-160 (2005)).

If CD28 is supporting myeloma cells survival, its activation *in vivo* is likely to be via direct cell contact with a $B7^+$ cell within the microenvironment. These include other $CD86^+$ myeloma cells and/or professional antigen presenting cell (APC) expressing CD80/CD86 (B cells, monocyte/macrophages, dendritic cells (DC)). Consistent with this, DC and other myeloid APC actively infiltrate implanted plasmacytomas in murine models (Corthay et al, Immunity 22:371-383 (2005)), and DC are readily found throughout myeloma infiltrates in patient bone marrow biopsies (Rettig et al, Science 276:1851-1854 (1997)).

The present invention results, at least in part, from studies designed to investigate whether CD28 can transduce survival signals to myeloma cells and whether MM CD28 is activated through cell-cell contact with other $B7^+$ cells. The invention provides methods of treating MM by targeting CD28 and/or CD28

signal transduction pathways, and compounds and compositions suitable for use in such methods.

SUMMARY OF THE INVENTION

The present invention relates, generally, to MM. More specifically, the invention relates treatment methods based on targeting the CD28 receptor and/or elements of the downstream CD28 signal transduction pathways. The invention further relates to methods based on blocking activation of CD28 resulting from direct contact between $CD28^+$ MM cells and $B7^+$ cells (e.g., $CD86^+$ myeloma cells and/or APC expressing CD80/CD86). The invention further relates to methods based on targeting dendritic cells (DC) expressing CD28 ligands, or otherwise disrupting myeloma:DC interaction.

Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C. CD28 and CD86 expression. Fig. 1A. CD28. The indicated cells were stained with anti-CD28 PE (filled histogram) or isotype control (open histogram) and analyzed by FACS. Top panels – cell lines. Bottom panels - primary myeloma isolates. Primary myeloma cells were purifed from 3 different patient samples (PS, bone marrow aspirates, relapsed MM) by CD138 immunomagnetic selection. Fig. 1B. Extramedullary plasmacytoma. Serial sections from an extramedullary plasmacytoma were stained with hematoxylin/eosin (left panel) or anti-human CD28 (brown staining, right panel). 200X magnification. Representative sections from one of two patients with intramuscular extramedullary plasmacytomas. Fig. 1C. CD80 and CD86. The cell lines indicated were stained with isotype control (open histograms), anti-

CD80, CD86 mAb, or CTLA4-Ig (closed histograms) and analyzed by FACS. Data is representative of 2 independent experiments.

Figures 2A-2D. Effect of CD28 activation on NFκB activation. Fig. 2A. IκBα and Bcl-x_L expression. 8226 or U266 cells were cultured with or without anti-CD28 mAb beads as indicated, and analyzed by Western blot using Ab specific for IκBα, Bcl-x_L or actin as indicated. Fig. 2B. Nuclear NFκB binding activity. 8226 or U266 cells were cultured with or without immobilized anti-CD28 mAb as indicated, and nuclear extracts analyzed by EMSA for binding to ^{32}P-labeled primers containing consensus NFκB binding sites. For supershift assays, samples were first incubated with no antibody (-), anti-p50, anti-Rel B, anti-c-Rel or anti-p65. Data is representative of 3 independent experiments. Fig. 2C. Binding to B7 does not induce NFκB signaling. U266 cells were treated as above except with or without CD28-Ig (100 μg/ml), and analyzed after 24h for nuclear NFκB binding activity. Data is representative of 2 experiments. Fig. 2D. Upregulation of Rel B expression by CD28 activation. U266 cells were treated with control or anti-CD28 mAb (soluble, 1 μg/ml) for 24h and analyzed for Rel B and actin expression by Western blot. Data is representative of 2 experiments.

Figure 3. CD28 activation inhibits myeloma cell proliferation. 2×10^5 8226, U266 and K562 (CD28-negative control) cells were cocultured with or without anti-CD28 mAb beads. After 24 hrs, cells were pulsed with ^{3}H TdR, and incorporation measured 18 hrs later and expressed as mean ± standard deviation of triplicate wells. Data shown is one experiment representative of 4 independent experiments.

Figures 4A-4C. CD28 activation protects against induced cell death. Fig. 4A. Serum starvation. The indicated myeloma cells were cultured in media (RPMI 1640) without serum (SS), or medium without serum plus soluble anti-CD28 mAb (SS+CD28). After 48 hrs, viability was determined by annexin V/PI staining. Data is the aggregate mean ± SD of 3 independent experiments. Fig. 4B. Dexamethasone-mediated cell death. The indicated myeloma cell lines were cultured in 0.1% FBS ± soluble anti-CD28 mAb and 100 μM dexamethasone. After 72 hrs, viability was determined by annexin V/PI staining. Data is the aggregate mean ± SD of 3 independent experiments. Fig. 4C. Primary myeloma cells. Primary myeloma cells were purified from 3 different patient samples (bone marrow aspirates, relapsed MM) CD138 immunomagnetic selection (Miltenyi Biotech). The samples were >90% CD138 positive and were all $CD28^{+}$. 2 x 10^5 input cells were cultured in 10% FCS (PS 1, left panel) or no serum (PS 1-3, right) plus/minus anti-CD28 mAb (9.3, 1 μg/ml) for 24h. Total viable cell numbers were then counted and expressed as the percentage of the starting input number of cells.

Figure 5. DC physically interact with myeloma cells. Bone marrow biopsy from patient 88 with relapsed MM was stained for CD138 for myeloma cells (brown staining cells, left panel, 400X) or fascin for DC (brown staining cells, right panel, 1000X). Photomicrograph of fascin-positive cells was taken from a region in the center of the field shown in the left panel. Arrows point to interdigitating DC dendrites.

Figures 6A-6E. Coculture with DC downmodulates MM proliferation and enhances survival. Fig. 6A. Proliferation. K562 cells were cultured in media alone (K562m, Km), or differentiated with PMA (K562p or Kp) or PMA+TNF-α

(K562 P+T or Kpt), irradiated and cocultured with 8226 cells (right panel) or U266 cells (left panel). Proliferation was measured by thymidine incorporation as above. Data is representative of 4 independent experiments. Fig. 6B. MM viability following coculture with DC. CFSE-labeled 8226 cells were cocultured with irradiated PMA-differentiated K562 cells at a 1:1 ratio. After 24 hrs, the cultures were stained with propidium iodide and analyzed by flow cytometry. Data is representative of 3 independent experiments. Fig. 6C. CD28-Ig blockade. DC were differentiated from KG1 using PMA, irradiated, and cocultured with 8226 cells. CD28-Ig was added to the DC 1 hr before addition of MM cells. Proliferation was measured by thymidine incorporation as above. Data is representative of 2 experiments. Fig. 6D. Immature vs. mature DC derived from normal monocytes. To generate immature DC, monocytes were cultured in GM-CSF and IL-4 for 8 days. To generate mature DC, TNF-α was added for the last four days of culture. These cells were then irradiated and cocultured with myeloma cells at the ratios indicated. Proliferation was measured by thymidine incorporation as above. Data is representative of 2 independent experiments. Fig. 6E. Protection against dexamethasone-induced cell death. 8226 cells were cultured alone, or 1:1 with irradiated, undifferentiated K562 cells (DC precursors) or K562- derived DC (DC), treated for 72h with 100 μM dexamethasone, and analyzed by FACS for 7AAD (dead cells) and CD28 expression (MM cells). % viable refers to myeloma cell viability. Data is representative of 2 independent experiments.

Figure 7. CD80 and CD86 expression in MM cell lines. The cell lines indicated were stained with either isotype control (open histograms) or anti-CD80 or CD86 mAb (PE conjugated, Immunotech) or CTLA4-Ig followed by GαH IgG

PE) (closed histograms). Cells were then analyzed by FACS. Data is representative of 2 independent experiments.

Figures 8A and 8B. Myeloma indoleamine 2,3 dioxygenase (IDO) expression. Fig. 8A. Constitutive. Cells were permeabilized (Cytofix-Cytoperm kit, BD Pharmingen), washed and stained with either isotype matched IgG (open histograms) or anti-IDO antibody (closed histograms). Samples were then analyzed by flow cytometry. Fig. 8B. Crosslinking of B7 by CTLA4Ig. U266 cells were cultured in media alone or plus CTLA4Ig (100 mM) for 24 hrs, permeabilized, washed and stained with either isotype matched IgG (open histograms) or anti-IDO antibody (closed histograms). Samples were then analyzed by flow cytometry.

Figure 9. Anti-CD28-toxin conjugates. Monoclonal antibody (mAb) 9.3 was biotinylated, and saporin conjugated to streptavidin (streptavidin-ZAP) added at equimolar concentrations. 5×10^5 of the indicated MM cell lines were cultured with streptavidin-ZAP alone, biotinylated mAb 9.3 alone or mAb 9.3-Strepavidin-ZAP at the concentrations indicated. Viable cell numbers were determined after 72h.

DETAILED DESCRIPTION OF THE INVENTION

The present invention results from studies identifying CD28 as a contributor to the pathogenesis of multiple myeloma. As shown in the Example below, activation of myeloma cell CD28 can induce activation of NFκB, down regulate MM cell proliferation and protect against serum starvation and dexamethasone-induced cell death. The invention provides therapeutic strategies designed to target CD28 directly, to target elements of the downstream CD28

signal transduction pathways and/or to modulate (e.g., block) interaction of CD28 with activating ligands (e.g., CD86). These strategies can be used to treat MM patients bearing $CD28^+$ myeloma cells, for example, by inhibiting outgrowth of $CD28^+$ myeloma cells in patients undergoing chemotherapy. These strategies can be used alone or in combination with other chemotherapies (e.g., with Rituxan and Herceptin, used alone or in combination).

In accordance with one embodiment of the invention, anti-CD28 antibodies can be used that kill $CD28^+$ myeloma cells directly (e.g., via antibody dependent cell-mediated cytotoxicity (ADCC)). Alternatively, anti CD28 antibodies can be used to target to $CD28^+$ cells an agent that can kill CD28 positive myeloma cells directly (e.g., a radioisotope or nonradioactive toxin, such as saporin, ricin,, calicheamicin or maytansinoid). Methods for conjugating such agents (toxins) to antibodies are well known in the art (see Example below).

Antibodies suitable for use in this embodiment (and the embodiments described below) include, but are not limited to, monoclonal antibodies and fragments thereof (e.g., Fab, Fab', $F(ab')_2$ fragments). Single chain antibodies can be used, as can chimeric antibodies, and humanized antibodies (Coligan et al, Current Protocols in Immunology, John Wiley & Sons Inc., New York, N.Y. (1994); Harlow, E. and Lane, D., Antibodies: A Laboratory Manual, Cold Spring Harbor Press (1988); Bedzyk et al, J. Biol. Chem., 265:18615 (1990); Chaudhary et al, Proc. Natl. Acad. Sci., 87:9491 (1990); USPs 4,946,778 and 5,359,046; European Patent Applications 125023, 171496, 173494, and 184187; WO 86/01533; PCT/US86/02269; Liu et al, Proc. Natl. Acad Sci. USA 84:3439-3443 (1987); Sun et al, Proc. Natl. Acad. Sci. USA 84:214-218 (1987); Better et al, Science 240:1041-1043 (1988); Maeda et al, Hum. Antibod. Hybrid. 2: 124-134 (1991); and Padlan, Mol. Immunol. 28: 489-498 (1991); Waldmann, Nature Medicine 9, 269-277 (2003)), and fragments thereof. Human or humanized antibodies are preferred. Examples anti-CD28 antibodies suitable for use in

treating MM include, but are not limited to, the 9.3 antibody, clone 28.2, MEM-233, 204.12 and L293 (further examples can be found at biocompare.com).

This embodiment of the invention includes pharmaceutical compositions comprising such antibodies (or antibody fragments). Such compositions can include other substances, such as pharmaceutically acceptable carriers. As used herein, a pharmaceutically acceptable carrier includes any and all solvents, including water, and the like that are not unacceptably toxic to the host. The antibodies/compositions of the invention can be administered to a MM patient in an amount sufficient to effect treatment. Suitable doses can be readily determined by one skilled in the art and can vary with the antibody (and toxin), the patient and the effect sought. This method of the present invention contemplates single as well as multiple administrations, given either simultaneously or over an extended period of time. Administration of an antibody can be carried out, for example, by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. Preferably, the antibodies of the invention are administered by injection, and, more preferably, subcutaneous, intraperitoneal, intra-arterial, or intravenous injection.

In accordance with another embodiment, the present invention relates to therapeutic strategies designed to target elements of the downstream CD28 signal transduction pathways. Examples of suitable targeting agents include immunosuppressive drugs, for example, those used in solid organ transplantations, such as the macrolide rapamycin, cyclosporin and FK506. These agents can be formulated using standard practices and appropriate administration regimes can be readily designed by one skilled in the art and can vary with the agent, the patient and the effect sought.

In accordance with a further embodiment, the present invention relates to therapeutic strategies designed to modulate (e.g., block) interaction of CD28 with its ligands (e.g., CD86) and thereby block CD28 activation. MM CD28 can be

activated by cell-cell interaction with DC and/or other CD86+ myeloma cells located in bone marrow. Therefore, included within this embodiment are strategies that inhibit or prevent such interaction. For example, in accordance with this embodiment, agents that bind to, but do not activate, CD28 can be used to block binding of the receptor with its ligand, as can agents that block the ligand. Examples of such agents include non-activating anti-CD 28 antibodies, or fragments (e.g., Fab or Fab'2 fragments) thereof or anti-CD80, CD86 antibodies, or fragments thereof. Chimeric soluble receptors that directly target or block the CD28 receptor itself (or its ligand(s)) can also be used (e.g., CTLA4-Ig or CD28-Ig). These agents can be formulated using standard practices and appropriate administration regimes can be readily designed by one skilled in the art and can vary with the agent, the patient and the effect sought.

In accordance with a further embodiment, the present invention relates to therapeutic strategies designed to modulate DC activation/function in the context of eliciting immune responses. Numerous factors (drugs, cytokines, microbial products) are known to have this modulating effect and can be expected to be suitable for use in modulating myeloma-DC interaction (examples of drugs include aspirin, thalidomide, steroids, vitamin D, prostaglandins, estrogen and progesterone; examples of cytokines include TNF-α, GM-CSF, IL-4 and FLT3 Ligand; examples of microbial products include lipopolysaccharide, fungal wall carbohydrates, and double-stranded RNA). Interestingly, thalidomide, which is thought to target the microenvironment in myeloma, has been shown to modulate DC function (Deng et al, J. Invest. Dermatol. 121:1060-1065 (2003); Mohty et al, J. Leukoc. Biol. 72:939-945 (2002)). Agents of this type can be formulated using standard practices and appropriate administration regimes can be readily designed by one skilled in the art and can vary with the agent, the patient and the effect sought.

Certain aspects of the invention can be described in greater detail in the non-limiting Example that follows.

EXAMPLE

Experimental Details

5 *Cell, reagents and culture*

RPMI 8226, U266, K562 and KG1 cell lines were obtained from American Type Culture Collection (Manassas, VA). The MM.1S cell line was the gift of Dr. S. Rosen (Robert H. Lurie Cancer Center). Early passage cells (i.e. in continuous culture for less than 2 months) were used for all experiments.
10 Primary myeloma cells were obtained from bone marrow aspirates from patients with relapsed myeloma (under IRB approved protocols) and purified by CD138 immunomagnetic selection (Miltenyi Biotec, Auburn, CA). The agonistic anti-CD28 mAb 9.3 (June et al, Mol. Cell Biol. 7:4472-4481 (1987)) was either used as soluble antibody (1 μg/ml), or immobilized to Dynal beads (Lake Success, NY)
15 and used at the indicated beads per cell ratio as previously described (Levine et al, Int. Immunol. 7:891-904 (1995)). For the serum starvation experiments, myeloma cell lines were cultured in media (RPMI 1640) without serum plus/minus anti-CD28 mAb, and viability determined by PI/annexin V staining and FACS analysis. Total viable cell numbers were enumerated by quadruplicate cell counts
20 using trypan blue. For dexamethasone treatment experiments, myeloma cell lines were cultured in 0.1% FBS $\pm$ anti-CD28 mAb + 100 μM dexamethasone, and viability assessed after 72 hrs.

Cell proliferation assays were done in 10% FCS. 0.5 μCi/well [*methyl*-^{3}H] thymidine was added for the final 18 hours of culture, and incorporation
25 measured using the Beta Plate scintillation counting system (Wallac Inc., Gaithersburg, MD) as previously described (Lindner et al, J. Immunol. 171:1780-

1791 (2003)). All conditions were performed in triplicates, and data is represented as the mean counts ± 1 SD.

Myeloma-DC coculture

5 K562 and KG1 were differentiated into dendritic cells/myeloid APC as previously described (Lindner et al, J. Immunol. 171:1780-1791 (2003); St. Louis et al, J. Immunol..162:3237-3248 (1999)). Briefly, cells were cultured in media alone or differentiated for 5-7 days with PMA (10 ng/ml, Sigma, St. Louis, MO) ± TNF-α (10 ng/ml, R&D Systems, Minneapolis, MN). Primary monocytes were
10 enriched from the peripheral blood MNC of normal donors (after informed consent under protocols approved by the University of Miami IRB) by plastic adherence and differentiated into DC using GM-CSF (1000 U/ml, Immunex, Seattle, WA) and IL-4 (1000 U/ml, R&D Systems, Minneapolis, MN) for 12 days ± TNF-α (20 ng/ml) for the last four days of culture as previously described
15 (Schlienger et al, Blood 96:3490-3498 (2000)). DC were then washed, irradiated at 3,000R (KG1, monocyte-derived DC) or 12,000R (K562) ^{137}Cs, and seeded into round-bottom wells alone or with myeloma cells at the cell numbers indicated. Cocultures were done in media + 10% FCS for the proliferation assays. After 24 hrs, wells were pulsed with [^{3}H] TdR, and incorporation was measured
20 for the next 18 hours of culture using Beta Plate scintillation counting system and expressed as mean ± standard deviation of triplicate wells. Where indicated, hCD28-Ig (R & D Systems) was added to the DC 1 hr before addition of MM cells.

The viability experiments were carried out in 0.1% FCS. Myeloma cells
25 were cultured alone, or 1:1 with irradiated, undifferentiated DC precursors or differentiated DC, treated for 72h with 100 μM dexamethasone, and analyzed by FACS for 7AAD (dead cells, Beckman Coulter) and CD28 expression (MM cells).

Flow cytometry

Cells were stained as previously reported (Lindner et al, J. Immunol. 171:1780-1791 (2003)) with anti-CD28, CD80, CD86 mAb or isotype control
5 (Immunotech, Westbrook, ME). Cells were also stained with CTLA4Ig (or isotype matched human Ig) and goat anti-human Ig PE. 10,000 live cells were analyzed by flow cytometry on a Coulter XL flow cytometer (Coulter, Hialeah, FL) using software supplied by the manufacturer.

10 *Western Blot*

Western blot analysis were performed as previously described (Lindner et al, J. Immunol. 171:1780-1791 (2003)). Briefly, cell lysates were made from 8226 or U266 cells were cultured with or without anti-CD28 mAb for 24 hrs. Cell lysates were made, protein levels quantitated by using the Micro BCA
15 reagent kit (Pierce, Rockford, IL) and equal amounts of protein were separated by SDS-PAGE (4% stacking/10% resolving), electroblotted to nitrocellulose, and probed with antibodies specific for Bcl-x_L (Boise et al, Immunity 3:87-98 (1995)), IκBα, or Rel B actin (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were visualized by chemoluminescent detection (ECL, Amersham Life Sciences,
20 Aylesbury, U.K).

Electromobility shift assay (EMSA)

EMSA's were carried out for NFκB family members as previously described (St. Louis et al, J. Immunol. 162:3237-3248 (1999)). Briefly, 8226 or U266 cells were cultured with or without anti-CD28 mAb for 24 hrs. Nuclear
25 extracts were made and equal amounts of protein were incubated with ^{32}P-labeled primer containing consensus NFκB binding sites (GAT CCA ACG GCA GGG GAA TTC CCC TCT CCT TA) and separated on 4% polyacrylamide gels. For

supershift assays, samples were first incubated with anti-Rel B, anti-p50, anti-p65, anti-c-Rel (all from Santa Cruz). Samples were visualized by autoradiography.

Immunohistochemistry

5 Biopsies of bone marrow and extramedullary plasmacytomas were obtained from patients with refractory/relapsed MM as part of IRB approved protocols and following informed consent. Consecutive sections were then stained with anti-CD138 (myeloma, R&D Systems), anti-fascin (D, Research Diagnostics Inc., Flanders, NJ), anti-CD28 (R&D Systems) or hematoxylin/eosin.

10

Results

Myeloma cell express CD28 and CD86.

As seen in Figure 1A (top panels), CD28 is abundently expressed on three human myeloma cell lines (RPMI 8226 (8226), MM.1S and U266) at levels
15 slightly lower than the cytotoxic T cell line YT. CD28 is also expressed on primary MM cells purified from bone marrow aspirates of patients with relapsed disease (Fig. 1A, lower panels), and also on the infiltrating myeloma cells from patients with extramedullary intramuscular plasmacytomas (Fig. 1B). As suggested by previous studies, all three MM cell lines also co-expressed CD86
20 but not CD80 (Fig. 1C), which is more clearly seen using indirect staining with CTLA4Ig.

CD28 triggers NFκB signaling in myeloma cells.

In T cells, one pathway downstream of the CD28 receptor is PI3K and Akt
25 activation (Frauwirth and Thompson, J. Clin. Invest. 109:295-299 (2002)). In myeloma cells, the pro-survival effects of IGF-1 are transduced through PI3K/Akt signaling and downstream NFκB activation (Tu et al, Cancer Res. 60:6763-6770 (2000); Mitsiades et al, Oncogene 21:5673-5683 (2002)). To assess whether

CD28 activation mimicks an IGF-1 signal, cellular IκBα was initially quantitated, following direct activation of CD28 by the agonistic anti-CD28 mAB 9.3, as IκB degradation is a central step in NFκB signal transduction. As seen in Figure 2A, antibody-mediated activation of CD28 alone results in substantial reduction of
5 cellular IκBα in both 8226 and U266 cells. The anti-apoptotic protein Bcl-x_L is not upregulated, consistent with previous reports that CD28 activation alone has no effect on Bcl-x_L expression in T cells (Boise et al, Immunity 3:87-98 (1995)). To assess CD28-induced NFκB signaling more directly, the levels of free NFκB dimers in the nucleus were measured by electromobility gel shift assay (EMSA).
10 As seen in Figure 2B, CD28 activation (using either bead bound or soluble mAb (not shown)) upregulates the total amount of nuclear NFκB binding in both U266 and 8226 cells. Supershift assays for specific NFκB family members demonstrate upregulation of p50, p65, Rel B and c-Rel, suggesting that both the canonical (p65, p50/p52 homodimers) and non-canonical (RelB) pathways are being
15 activated. In contrast, binding of CD86 with CD28Ig has no effect on NFκB signaling (Fig. 2C) – indicating that the effects of mAB 9.3 are not paradoxically due to the blocking of a myeloma CD28-myeloma CD86 interaction. Finally, it has been shown previously that NFκB signaling plays the primary role in upregulating expression of the NFκB family member Rel B (Cejas et al, Mol. Cell
20 Biol. 25:7900-7916 (2005)), and similarly found that CD28 activation induces Rel B expression in myeloma cell lines (Fig. 2D). Together, these findings support the conclusion that CD28 activation by itself in multiple myeloma cells results in NFκB signaling.

25 *CD28 downmodulates myeloma cell proliferation.*

The ability of CD28 to co-stimulate T cell proliferation and survival can be segregated into 2 independent downstream signaling pathways, with PI3K being essential for survival and factors binding the C-terminal proline motifs

being essential for proliferation and cytokine responses (Burr et al, J. Immunol. 166:5331-5335 (2001)). While CD28-mediated activation of NFκB in myeloma cells suggests that the PI3K pathway/survival is intact, in contrast to T cells, CD28 activation suppresses 8226 and U266 cell proliferation without having any
5 effect on the CD28-negative cell line K562 (Fig. 3).

CD28 activation enhances myeloma cell survival

Activation of NFκB by IGF-1, IL-6 and other stimuli has been shown to be a pro-survival signal in myeloma cells (Hideshima et al, J. Biol. Chem.
10 277:16639-16647 (2002)), suggesting that CD28 activation may also support myeloma cell survival. As serum contains sufficient IGF-1 to prevent apoptosis (Harrington et al, EMBO J. 13:3286-3295 (1994)), the question addressed was whether withdrawal of serum would induce cell death in 8226 and MM.1S cells, and whether CD28 activation could provided substitute survival signals. As seen
15 in Figure 4A, serum starvation results in significant cell death in both 8226 and MM.1S cells by 48 hrs. This death could be abrogated in part by the addition of anti-CD28 mAb, resulting in a two-fold increase in viability versus media alone.

To examine protection against a more defined death signal induced by a clinically relevant agent, a determination was made as to whether CD28 activation
20 could protect against dexamethasone (dex)-induced apoptosis. To minimize the possibility of serum IGF-1 masking a CD28 pro-survival signal, these assays were conducted in low serum conditions (0.1%, which alone did not affect the viability of the cell lines). As seen in Figure 4B, dexamethasone effectively killed all 3 MM cell lines tested, and anti-CD28 mAb-mediated activation resulted in
25 substantial protection against this dexamethasone-induced death.

Whether CD28 activation also had pro-survival effects in primary myeloma cells was examined in cells purifed from from 3 patient samples (relapsed disease), with the resulting cell populations >90% positive for plasma

cell marker CD138 (all 3 samples were CD28 positive). The purified MM cells were then cultured in 10% serum (Fig. 4C, left, for patient sample 1) or without serum (Fig. 4C, right) plus/minus anti-CD28 mAb for 24h. While primary myeloma cells faired reasonably well in 10% FCS (and were largely unaffected
5 by anti-CD28 mAb – which also demonstrates that the mAb was not inducing myeloma cell proliferation), there was a considerable loss of viable cells when serum was withdrawn. Consistent with the cell line findings, activation of CD28 significantly improved myeloma cell survival (doubling the viable cell numbers, which is in the same range seen for the cell lines).
10

DC associate with myeloma cells in vitro and in vivo

It seems likely that the biologically relevant activation of CD28 in myeloma cells occurs the same way it does in T cells, namely by engagement of CD80/CD86 during direct cell-cell contact with $B7^+$ cells. A predominant role for
15 $CD80/CD86^+$ bone marrow resident DC is favored, given the known interaction between normal plasma cells and DC as well as the exceptional potency of DC to activate T cells, suggesting that they have the requisite elements for efficient cell-cell interactions. This does not, however, exclude the possibility that MM cells expressing both CD28 and CD86 may, in part, support their own survival through
20 a myeloma-myeloma interaction. To assess a possible DC-MM interaction, an examination was first made of whether DC could be found in close physical proximity to myeloma cells in bone marrow biopsies from patients, as others have reported (Rettig et al, Science 276:1851-1854 (1997); Said et al, Blood 90:4278-4282 (1997)). CD138 staining was used to specifically identify myeloma cells,
25 and positivity for the actin bundling protein fascin plus morphology to histologically identify bone marrow DC (as previously reported (Rettig et al, Science 276:1851-1854 (1997)). In immunohistochemical studies of paraffin-embedded tissue, fascin expression has been found to be very specific for

dendritic cells (Bros et al, J. Immunol. 171:1825-1834 (2003); Pinkus et al, Am. J. Clin. Pathol. 118:335-343 (2002); Pinkus et al, Am. J. Pathol. 150:543-562 (1997)) and superior to HLA-DR or S100 staining for distinguishing DC from tissue macrophages (Vakkila et al, Pediatr. Dev. Pathol. 8:43-51 (2005)). It was found that 4 of 5 patients with relapsed myeloma had numerous fascin$^+$ cells with DC morphology within the marrow CD138$^+$ (myeloma) cell infiltrates, while the fifth patient (pt. 90) had fewer but detectable numbers of DC (Table 1). These fascin$^+$ cells were predominantly localized within the CD138$^+$ cell infiltrates, as non-infiltrated areas of bone marrow within the same marrow section had significantly fewer fascin$^+$ cells (except for patient 90, who had the same (low) number in both areas). Figure 5 demonstrates the numerous fascin$^+$ cell processes and less frequent cell bodies (right) that could be readily found interdigitating between CD138$^+$ myeloma cells (left). Because fascin expression positively correlates with DC maturation (Vakkila et al, Peadiatr. Dev. Pathol. 8:43-51 (2005)), these findings suggest that myeloma-infiltrating DC have the high CD80/CD86 expression that is typical of mature DC. Consistent with this, CD83 staining yields similar findings (not shown).

Table 1

	Fascin positive cells (average ± SD)	
Patient ID	CD138$^+$ infiltrated	Non-infiltrated
87	14.6 ± 1.9	0.8 ± 0.8
88	19.4 ± 2.1	0.4 ± 0.8
89	15.4 ± 1.9	0.2 ± 0.4
90	2.2 ± 1.2	1.4 ± 0.8
91	15.8 ± 1.7	0.2 ± 0.4

Fascin-positive cells within CD138$^+$ cell infiltrated and non-infiltrated areas. Consecutive sections from bone marrow biopsies from 5 patients with relapsed multiple myeloma were stained with CD138 or fascin. Areas with CD138$^+$ cell infiltrates were first identified, and infiltrated (myeloma) and non-infiltrated areas (normal) were then reexamined for fascin staining on serial sections. The number of fascin-positive cell bodies with dendritic projections (excluding capillary endothelial cells) were counted per high powered field (1000X), and averaged for 5 fields.

Coculture with DC modulates myeloma cell proliferation and survival

To characterize the possible effects of DC interaction on myeloma cells, myeloid DC derived from human $CD34^+$ leukemia cell lines (KG1 (St. Louis et al, J. Immunol. 162:3237-3248 (1999) and K562 (Lindner et al, J. Immunol. 171:1780-1791 (2003)) were used initially. It has been shown that these myeloid blasts are not immunostimulatory in their undifferentiated state, but undergo differentiation in response to cytokines or phorbol esters (PMA) to antigen-presenting cells that have characteristic DC markers (MHC I, II, CD40, CD80, CD86, CD83), expression of DC-specific molecular markers (e.g. DC-CK1, DC-STAMP) and unique DC function (e.g. the ability to cross-present antigen, potently activate T cells to a significantly greater degree compared to normal monocytes, etc) (Lindner et al, J. Immunol. 171:1780-1791 (2003); St. Louis et al, J. Immunol. 162:3237-3248 (1999); Ackerman and Cresswell, J. Immunol. 170:4178-4188 (2003); Li et al, Cell Immunol. 227:103-108 (2004); Hajas et al, Immunol. Lett. 92:97-106 (2004); Suciu-Foca et al, Hum. Immunol. 62:1065-1072 (2001); Hulette et al, Arch. Ermatol. Res. 293:147-158 (2001); Wang et al, Immunity 15:971-983 (2001); Soilleux et al, J. Immunol. 165:2937-2942 (2000)). Cell-line derived DC are a more homogenous population than primary monocyte-derived DC, with less variability due to progenitor purity, maturation differences, etc. A first characterization was first made of the effect on myeloma cell proliferation. Proliferation of 8226 cells (Figure 6A, left panel) and U266 cells (Figure 6A, right panel) was not affected when cocultured with irradiated, undifferentiated K562 cells . However, similar to activation with anti-CD28 mAb, coculture with K562 cells -differentiated to DC by PMA or PMA+TNF-α (TNF addition drives further DC maturation) significantly downmodulated the proliferation of 8226 and U266 cell proliferation. KG1 yielded the same results

(below). It is unlikely that this decrease is due residual PMA carryover as PMA carryover has not been detected in other sensitive assays (e.g., T cell proliferation) and the finding that PMA alone has no effect on 8226 or U266 cell proliferation (data not shown and Zhang et al, Leukemia 12:610-618 (1998)). To formally exclude the possibility that the inhibition of proliferation was actually due to the myeloma cells being killed, 8226 cells were labeled with the membrane dye CFSE and assayed for cell viability (propidium iodide exclusion) after coculture with K562-derived DC. As seen in Figure 6B, after 24 hrs of coculture, there are very few PI-positive dead cells in either the $CFSE^+$ 8226 or $CFSE^-$ K562-DC populations.

It is likely that a DC-MM interaction involves multiple receptor-ligand bindings, some of which could mediate the same responses as seen for CD28. To assess the contribution of CD28 on the modulation of proliferation by this interaction, CD80/CD86 was blocked with the chimeric CD28 receptor-immunoglobulin Fc molecule CD28-Ig. As seen in Figure 6C, addition of CD28-Ig reversed the inhibition of 8226 cell proliferation induced by co-culture with DC derived from KG1. Similar results were obtained with U266 and MM.1S cells (not shown).

Although it has been shown previously that these leukemia-derived DC have all the phenotypic and functional characteristics of DC derived from normal progenitors, it is possible that these DC have aberrant properties (compared to DC derived from normal progenitors) in regards to interaction with plasma/myeloma cells. To address this question, 8226 cells were co-cultured with DC differentiated from normal peripheral blood monocytes using a well characterized cytokine combination (Schlienger et al, Blood 96:3490-3498 (2000)). In addition, the question of whether immature DC (iDC, differentiated with GM-CSF + IL-4) affected myeloma cell proliferation differently than mature DC (mDC, GM-CSF +IL-4 + TNF-α) was addressed. Immature DC have lower expression of

costimulatory ligands than mature DC, and are significantly less effective at activating T cells (Banchereau and Steinman, Nature 392:245-252 (1998)). If these same molecules are important in regulating myeloma proliferation, it would be predicted that mDC would be more potent than iDC. As seen in Figure 6D, both iDC and mDC are capable of significantly downmodulating 8226 cell proliferation at cell ratios of 1:1. However, mDC appear to be more potent in this regard as they can do this at lower DC:myeloma cell ratios than iDC. The differential ability of mature DC vs. immature DC to do this parallels a similar difference in the ability to activate T cells, suggesting that the level of DC costimulatory ligand expression may also directly regulate the downmodulation of MM cell proliferation. Together, these data demonstrate that DC derived from normal and leukemic progenitors can similarly downmodulate MM proliferation.

To assess whether DC can also transduce a survival signal similar to anti-CD28 mAb, 8226 cells were cultured alone, with undifferentiated K562 cells, or K562-derived DC in 100 μM dexamethasone (Fig. 6E). Similar to the results with antibody-mediated activation, co-culture with DC doubles the viability of 8226 vs. myeloma cells alone or plus DC precursors.

Myeloma cells co-express CD86 and express indoleamine 2, 3 dioxygenase (IDO)

The finding that activation of CD28 is qualitatively different in myeloma cells versus T cells raised the possibility that the effect on the other side of the interaction, namely the $B7^+$ cell, was also different. The precedent for this in T cells is the CD28 family member CTLA4, which also binds to both CD80 and CD86. CTLA4 signaling does not activate T cells but rather inhibits IL-2 secretion while upregulating the anti-apoptotic molecule Bcl-x, resulting in a viable but unresponsive state (Blair et al, J. Immunol. 160:12 (1998)). Moreover, CTLA4-mediated crosslinking of B7 induces DC to express IDO, which results in T cell anergy. As noted above, constitutive expression of CTLA4 in myeloma

cells has not been detected , although an examination has not been made as to whether it can be induced by CD28 activation as has been reported for T cells (Algre et al, J. Immunol. 157:4762 (1996)). It is hypothesized that myeloma CD28, which also does not induce the secretion of immunostimulatory IL-2 (Shapiro et al, Blood 98:187 (2001)), may be functioning like CTLA4 in triggering IDO expression and generating a locally immunosuppressive environment. In the present model, there are two potential IDO-secreting B7$^+$ partners, the myeloma cells themselves and/or dendritic cells.

Studies have been initiated to examine IDO expression in the present system. As part of the studies of the potential "autocrine" CD28-CD86 interaction between myeloma cells, CD86 expression in MM cell lines has been examined initially. As seen in Figure 7, all three CD28$^+$ MM cell lines are CTLA4-Ig positive, expressing CD86 but not CD80. Expression in MM1.S is low, but has been confirmed by microarray analysis (not shown).

As shown in Figure 8A, all three MM cell lines also constitutively express IDO (living in their own private IDO, so to speak), whereas the myeloid leukemia cell line KG1 does not. Whether this expression is due to interaction between myeloma CD28 and CD86 is under study - these studies being technically less straightforward given that most of the available blocking agents (e.g. CTLA4Ig, anti-CD86 mAb) can also crosslink CD86. This is shown in Fig. 8B, demonstrating that CTLA4-Ig treatment of U266 upregulates IDO expression over baseline, consistent with the possibility that there is a B7 inducible component to IDO expression in the myeloma cell lines. Studies examining the effect of this expression on T cell activation are also underway, as are experiments assessing whether myeloma cells can induce IDO expression in DC. Although preliminary, these finding offer a second potential mechanism as to why CD86$^+$ myelomas have a worse prognosis. Further, this suggests an overall model where CD28 mediates direct pro-survival effects in the myeloma cells as well an

indirect immunosuppressive effect through CD86 signaling on the "stromal" partner.

Toxin conjugated anti-CD28 mAb kills myeloma cell lines:

5 If CD28 plays an important role in MM cell survival, its expression cannot be easily downregulated – making the receptor itself an attractive target. One approach is to use anti-CD28 mAb to elicit antibody-dependent cell-mediated cytotoxicity (ADCC) but it is possible that MM cells are resistant to ADCC. To overcome this, the anti-CD28 mAb 9.3 has been conjugated to the toxin saporin.
10 Saporin is a ribosomal inactivating protein from the plant *Saponaria officinalis* that has no cell binding properties of its own but when conjugated to antibodies (as a means to gain entry by endocytosis) is a very potent toxin (Siena et al, Blood 72:756 (1988)). Saporin-antibody conjugates are routinely used in the neuroscience to ablate specific neurons *in vivo* (Sherren et al, Neuroscience
15 133(2):485-92 (2005)) and have been used to kill myeloma cells *in vitro* at sub-nanomolar concentrations (Vooijs et al, Can. Immunol. Immunother. 42:319 (1996)). Monoclonal antibody 9.3-saporin conjugates have been generated by adding equimolar concentration of saporin conjugated to streptavidin (streptavidin-ZAP, BioCore-Advanced Targeting Systems) to biotinylated mAb
20 9.3 (streptavidin binds with extremely high affinity to biotin ($Ka=10^{15}\ M^{-1}$) that is essentially irreversible). As seen in Figure 9, while neither saporin or mAb 9.3 alone prevented the expansion of viable MM cell numbers over 72h, the mAb 9.3-saporin conjugate could modestly (U266) to completely (8266) eradicate the target myeloma cells. This demonstrates the availability of a CD28-specific agent
25 that can be tested against normal cells (e.g., T cells) and in the *in vivo* murine model.

Summarizing, CD28 activation by itself induces NFκB signaling in myeloma cells, and delivers both anti-proliferative (which may protect against chemotherapy) and pro-survival signals. This is consistent with the clinical observation that CD28 expression on myeloma cells correlates significantly with prognosis and disease progression, suggesting that CD28 positivity is selected for under treatment pressure. Although not described for myeloma cells, activation of CD28 has been shown to significantly augment NFκB activation in mitogen-treated T cells via activation of IKK (Harhaj and Sun, J. Biol. Chem. 273:25185-25190 (1998)), while super-agonistic anti-CD28 antibodies alone can activate NFκB signaling in the absence of a TCR signal (Luhder et al, J. Exp. Med. 197:955-966 (2003)). It is likely that the intracellular signaling pathway from the CD28 receptor to NFκB activation (CD28→PI-3 kinase→PDK-1→Akt→IκB) is the same in myeloma cells as it is in T cells, since the p85 subunit of PI-3 kinase associates with CD28 in both cell types following binding to CD80 (Rudd and Schneider, Nat. Rev. Immunol. 3:544-556 (2003); Zhang et al, Leukemia 12:610-618 (1998)). PI-3K activation of Akt and NFκB is also seen in myeloma cells treated with IGF-1 (Tu et al, Cancer Res. 60:6763-6770 (2000); Mitsiades et al, Oncogene 21:5673-5683 (2002)), an established survival factor for myeloma, and further supports a similar survival signal transduced by CD28 activation. Interestingly, the mammalian target of rapamycin (mTOR) is a central downstream component of Akt signaling, and inhibition by rapamycin both inhibits CD28-mediated mTOR activation in T cells (Ghosh et al, Blood 99:4517-4524 (2002)) and sensitizes cells to dexamethasone-induced apoptosis in myeloma (Stromberg et al, Blood 103:3138-3147 (2004)). It is also possible that CD28 in myeloma cells is activating NFκB through additional pathways delineated in T cells, such as VAV→MEKK1 (Rudd and Schneider, Nat. Rev. Immunol. 3:544-556 (2003)). Finally, NFκB itself (separate from the upstream signaling pathway) has been clearly shown to play an important role in myeloma

survival (Hideshima et al, J. Biol. Chem. 277:16639-16647 (2002); Mitsiades et al, 99:4079-4086 (2002)). Together, the intracellular signaling pathways of CD28 are consistent with a pro-survival function of this receptor in myeloma.

Despite the similarities in intracellular signal transduction pathways, significant differences exist in CD28 activation in myeloma cells versus T cells. First, a synchronous antigen receptor "signal 1" that is clearly required for CD28 costimulation in T cells is not necessary in myeloma cells (at least *in vitro*). Myeloma cells do not express the B cell receptor, and attempts to define an alternative signal 1 (e.g. IL-6, PKC agonists (Shapiro et al, Blood 98:187-193 (2001); Pope et al, Blood 96:1274-1279 (2000)) that can be costimulated by CD28 have been equivocal. However even in T cells, CD28 can signal in the absence of a concurrent TCR signal (Rudd and Schneider, Nat. Rev. Immunol. 3:544-556 (2003); LeBlanc et al, Blood 103:1787-1790 (2004)), and super-agonistic anti-CD28 antibodies can activate T cells without a signal 1 (Luhder et al, J. Exp. Med. 197:955-966 (2003)). Thus, it is possible that CD28 activation alone can induce cellular responses in myeloma cells because it is triggered at a lower threshold than in T cells. Alternatively, there may be less negative regulation of downstream signaling pathways (e.g. PI-3K by PTEN) in myeloma vs. T cells, resulting in transduction of a comparatively larger signal. Finally, it is possible that a yet undescribed signal 1 is present in culture, although the ability of anti-CD28 mAb to rescue MM cells in serum free conditions suggests that it is not an exogenous soluble factor. It is important to reiterate that normal plasma cells also express CD28 (Kozbor et al, J. Immunol. 138:4128-4132 (1987); Robillard et al, Clin. Cancer Res. 4:1521-1526 (1998)), so it is likely that the effect of CD28 in myeloma cells recapitulates normal immunobiology rather than being some aberrancy of transformation.

A second difference between myeloma and T cells is the effect of CD28 on proliferation, with augmentation in T cells and downmodulation in myeloma

cells. The effect on MM cell proliferation reported is consistent with previous studies demonstrating that soluble anti-CD28 mAb 9.3 (the same as used in above studies) could suppress the proliferation of the MER myeloma cell line by 50% (Kornbluth, Curr. Top. Microbiol. Immunol. 194:43-49 (1995)), as well as a recent study using another anti-CD28 mAb (Qiu et al, Cell Immunol. 236(1-2):154-160 (2005)). Although the reasons for this difference are currently unclear, it has been shown in T cells that the ability of CD28 to augment proliferation and survival can be segregated into 2 downstream signaling pathways (Burr et al, J. Immunol. 166:5331-5335 (2001)). It is possible that myeloma cells lack the downstream pathways involved in proliferation while retaining the pro-survival pathway, and pro-survival signals by themselves can negatively regulate cell cycle progression (Hazlehurst et al, Oncogene 19:4319-4327 (2000); Landowski et al, Oncogene 22:2417-2421 (2003); Linette et al, Proc. Natl. Acad. Sci. USA 93:9545-9552 (1996)). Another possibility is that CD28 in myeloma cells does not elicit autocrine secretion of proliferative cytokines, whereas CD28-induced autocrine secretion of IL-2 is a major factor driving T cell proliferation. Although a decrease in MM cell proliferation would seem to lead to less aggressive disease, decreased proliferation would increase resistance to chemotherapeutic agents that are most effective against cycling cells. In these aspects, the response to CD28 activation is very similar to the response of myeloma cells following adhesion to extracellular matrix proteins - namely induction of NFκB, cell cycle arrest, and enhanced survival/resistance to chemotherapeutic agents (Damiano et al, Blood 93:1658-1667 (1999); Hazlehurst et al, Oncogene 19:4319-4327 (2000); Landowski et al, Oncogene 22:2417-2421 (2003)).

In addition to defining differences between myeloma and T cells the present findings also stand in contrast to some previous studies in myeloma cells where variable effects of CD28 activation on proliferation and survival have been

found, including a recent report of the induction of MM cell apoptosis by anti-CD28 mAb (Qiu et al, Cell Immunol. 236(1-2):154-160 (2005)). There are potentially several technical variables that may underlie these differences. First, the different anti-CD28 antibodies used differ in binding, resulting in an overall agonistic or antagonistic (by blocking possible CD28-CD86 interactions between MM cells) effect. Second, the survival studies were done in no/low serum conditions, whereas previous studies were done in 10% serum. It is possible that in the higher serum conditions, the level of exogenous pro-survival factors (especially IGF-1) were sufficient to mask any effect of CD28 activation on survival.

If CD28 is supporting myeloma cell survival, activation *in vivo* must be occurring through direct contact with CD80/CD86-positive cells. It is believed that there are two, non-mutually exclusive possibilities – binding to CD86 on other myeloma cells, and myeloma cell interaction with normal professional antigen presenting cells (APC). Regarding a possible myeloma-myeloma interaction, the aggregate findings of several studies is that >50% of primary myeloma isolates are CD86 positive, 100% of $CD28^+$ myelomas are $CD86^+$, and that $CD86^+$ myelomas have a significantly worse prognosis (Robillard et al, Clin. Cancer Res. 4:1521-1526 (1998); Pope et al, Blood 96:1274-1279 (2000)). Similarly, it is shown above, and it has been shown elsewhere that myeloma cell lines typically co-express CD28 and CD86, although these latter two reports are contradictory as to whether autocrine CD28 activation is occurring. The second possibility is that CD28 activation on MM cells occurs the same way it does on T cells, namely by cell to cell contact with APC expressing CD80/CD86 (B cells, monocyte/macrophages and DC). In addition to B7 expression, professional APC have specialized ability to directly interact with other immune cells that includes the expression of appropriate adhesion molecules and chemoattractant chemokines. This is particularly true for DC (Banchereau and Steinman, Nature

392:245-252 (1998)). Although not reported for myeloma cells, there is considerable evidence that DC are directly involved in the survival, proliferation and differentiation of normal B cells and plasma cells. These include DC expression of IL-6 (Zhou and Tedder, Blood 86:3295-3301 (1995); Santiago-Schwarz et al, Stem Cells 14:225-231 (1996)), that DC and B cells form clusters *in vitro* and *in vivo* (Kushnir et al, J. Immunol. 160:1774-1781 (1998)), that this direct interaction provides B cells with proliferation and survival signals (Wykes and MacPherson, Immunology 100-1-3 (2000)), and drives their differentiation to plasma cells (Fayette et al, Scand. J. Immunol. 48:563-570 (1998); Dubois et al, J. Immunol. 161:2223-2231 (1998); Dubois et al, J. Immunol. 162:3428-3436 (1999)). Recent studies have shown that dendritic cells enhance plasmablast survival and differentiation, in part through secretion of APRIL and/or BAFF (Balazs et al, Immunity 17:341 (2002)). It is likely that such an advantageous interaction would be retained by transformed plasma cells, and is consistent with: 1) substantial numbers of host dendritic cells (and other APC) rapidly infiltrate implanted plasmacytomas (Corthay et al, Immunity 22:371-383 (2005)), 2) in primary patient isolates, bone marrow DC are selectively and intimately associated with myeloma cells (the above-reported finding and Rettig et al, Science 276:1851-1854 (1997)), and 3) exogenously added APRIL and BAFF protect myeloma cells against apoptosis caused by IL-6 withdrawal and dexamethasone (Moreaux et al, Blood 103:3148-3157 (2004)). The present results indicate that one of the molecular components of a DC-MM interaction is activation of CD28, but given the molecular complexity of DC interactions with other immune cells, it is likely that other important signals (e.g. integrin-mediated signals) are also transduced to myeloma cells by this contact.

* * *

All documents and other information sources cited above are hereby incorporated in their entirety by reference.

WHAT IS CLAIMED IS:

1. A method of treating multiple myeloma (MM) comprising administering to a patient in need thereof an amount of an agent that targets CD28 or an element of a downstream CD28 signal transduction pathway, or of an agent that modulates interaction of CD 28 with an activating ligand, sufficient to effect said treatment.

2. The method according to claim 1 wherein said patient bears CD28+ myeloma cells.

3. The method according to claim 2 wherein said patient is undergoing chemotherapy and said method inhibits outgrowth of said CD28+ myeloma cells.

4. The method according to claim 1 wherein said agent is an anti-CD28 antibody or fragment thereof.

5. The method according to claim 4 wherein said antibody, or fragment thereof, kills CD28+ myeloma cells.

6. The method according to claim 4 wherein said antibody, or fragment thereof, targets to CD28+ myeloma cells a substance that kills said CD28+ myeloma cells.

7. The method according to claim 6 wherein said substance is a radioisotope or nonradioactive toxin.

8. The method according to claim 7 wherein said substance is a nonradioactive toxin selected from the group consisting of saporin, ricin, calicheamicin and maytansinoid.

9. The method according to claim 1 wherein said agent targets an element of a downstream CD28 signal transduction pathway.

10. The method according to claim 9 wherein said agent is an immunosuppressive agent.

5 11. The method according to claim 1 wherein said agent modulates interaction of CD28 with an activating ligand and thereby blocks CD28 activation.

12. The method according to claim 11 wherein said agent is a non-activating anti-CD28 antibody or fragment thereof.

10 13. The method according to claim 11 wherein said agent is a soluble receptor that blocks the CD28 receptor or a CD28 ligand.

14. A method of treating MM comprising administering to a patient in need thereof an amount of an agent that modulates myeloma-dendritic cell (DC) interaction sufficient to effect said treatment.

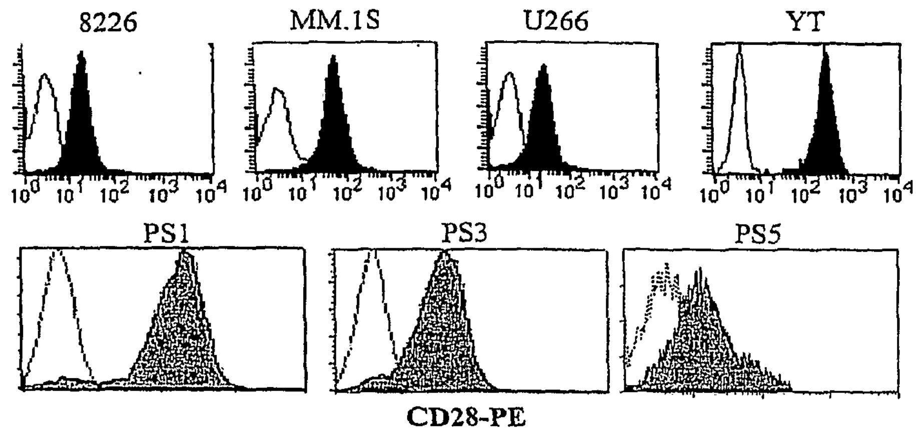
15 15. A composition comprising an anti-CD28 antibody, or fragment thereof, and chemotherapeutic agent.

16. The composition according to claim 15 wherein said chemotherapeutic agent is Rituxan or Herceptin.

17. The composition according to claim 15 wherein said antibody is
20 9.3 antibody, clone 28.2.

Figure 1.

A.



B.

C.

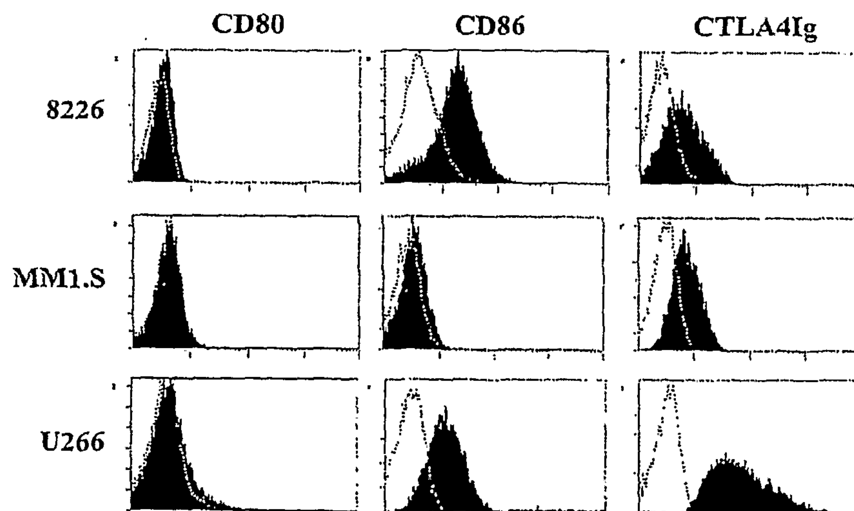
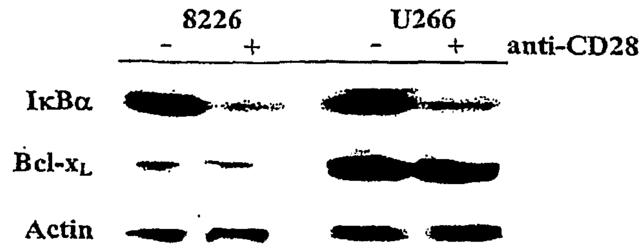
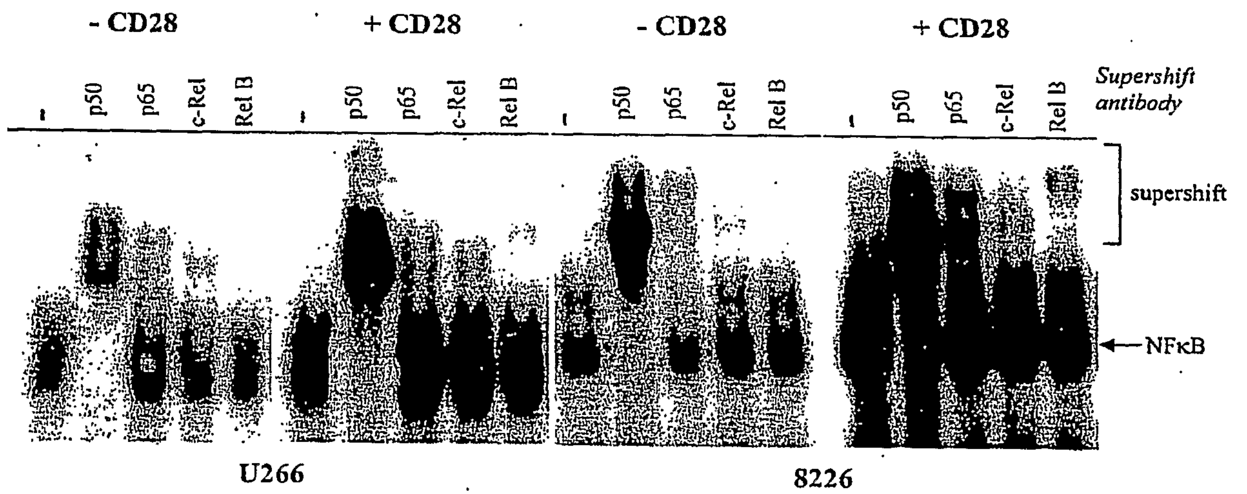


Figure 2.

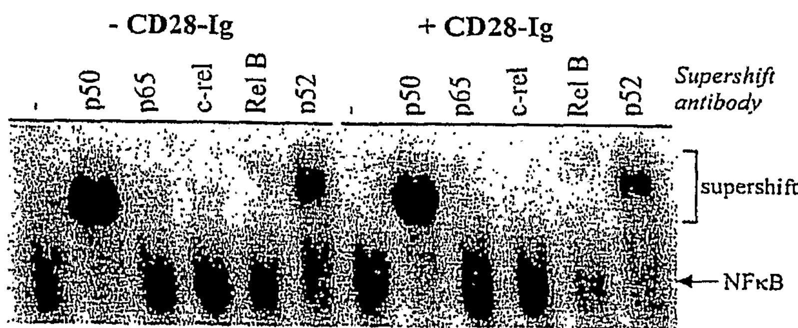
A.



B.



C.



D.

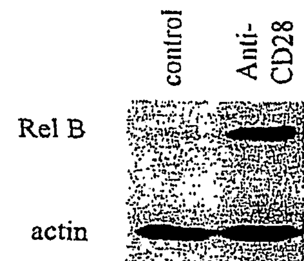


Figure 3.

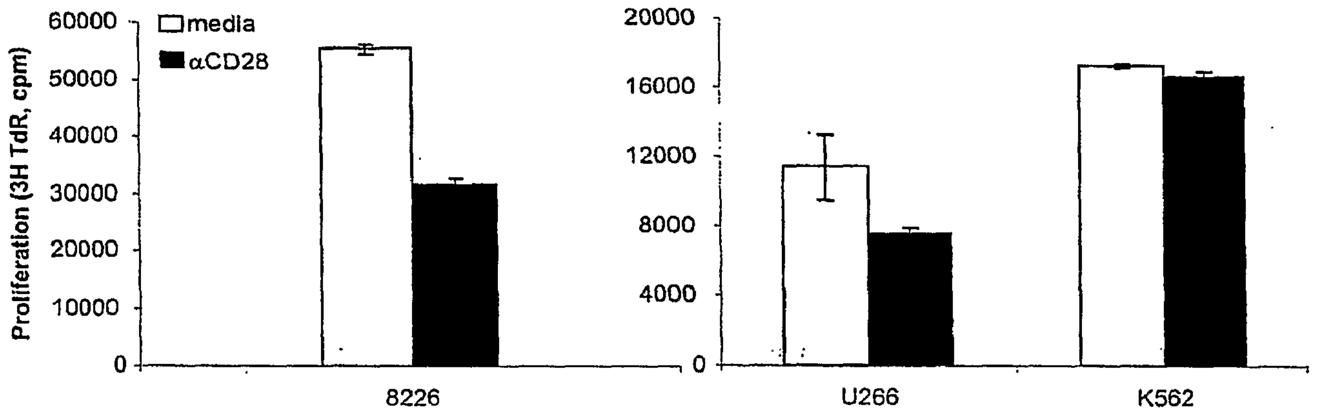
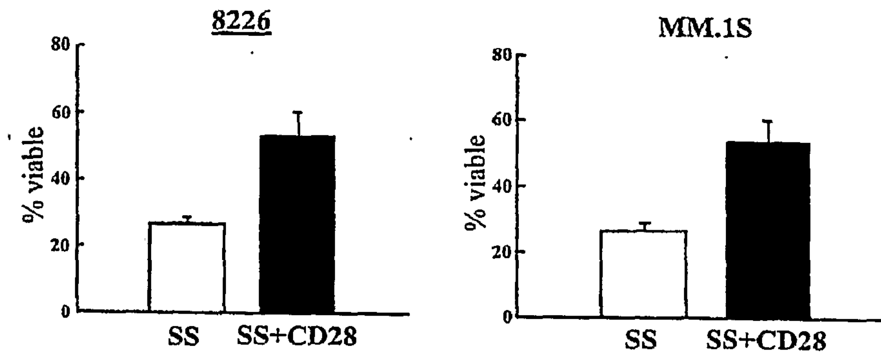
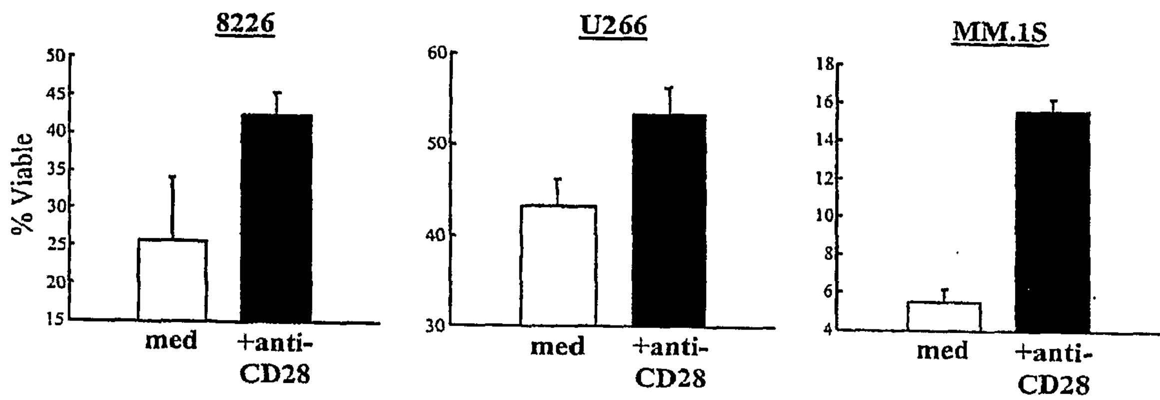


Figure 4.

A.



B.



C.

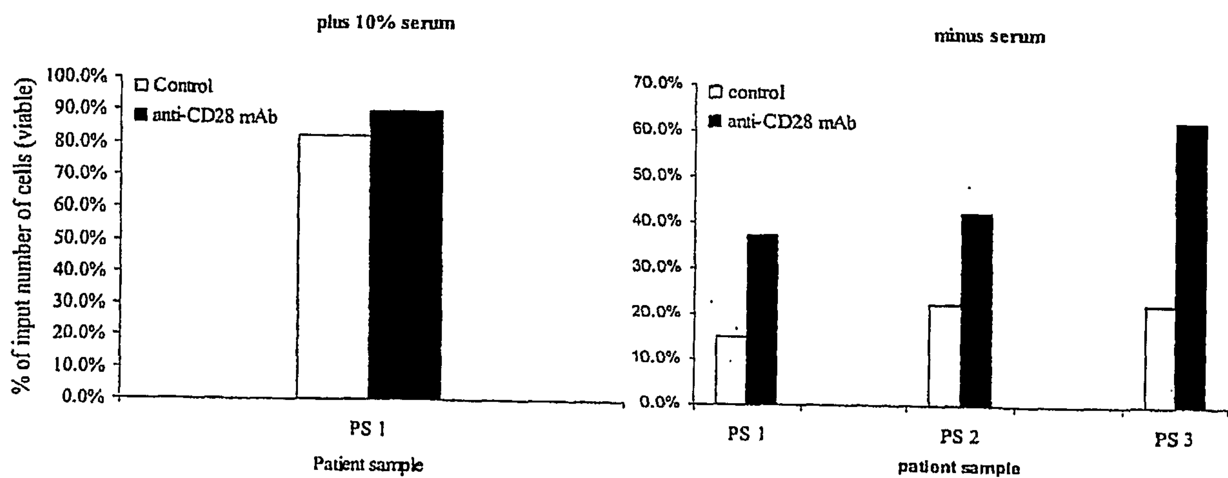
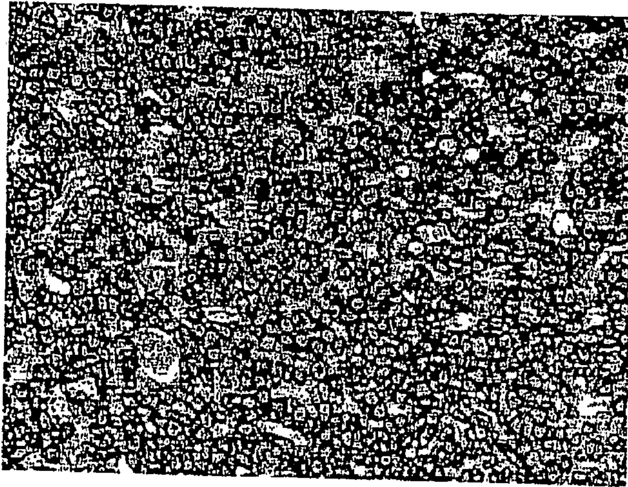


Figure 5.



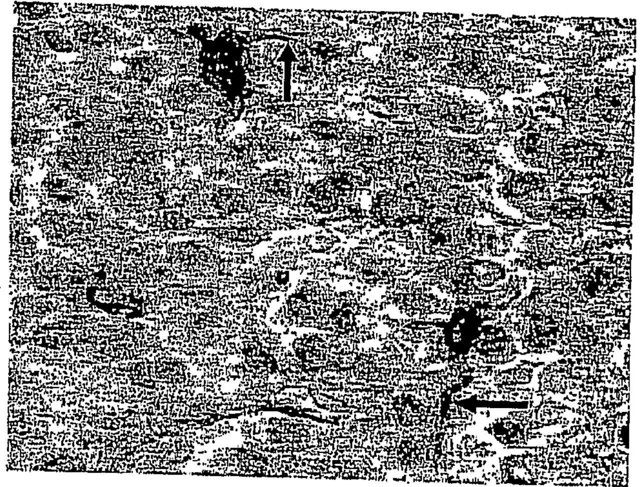


Figure 6.

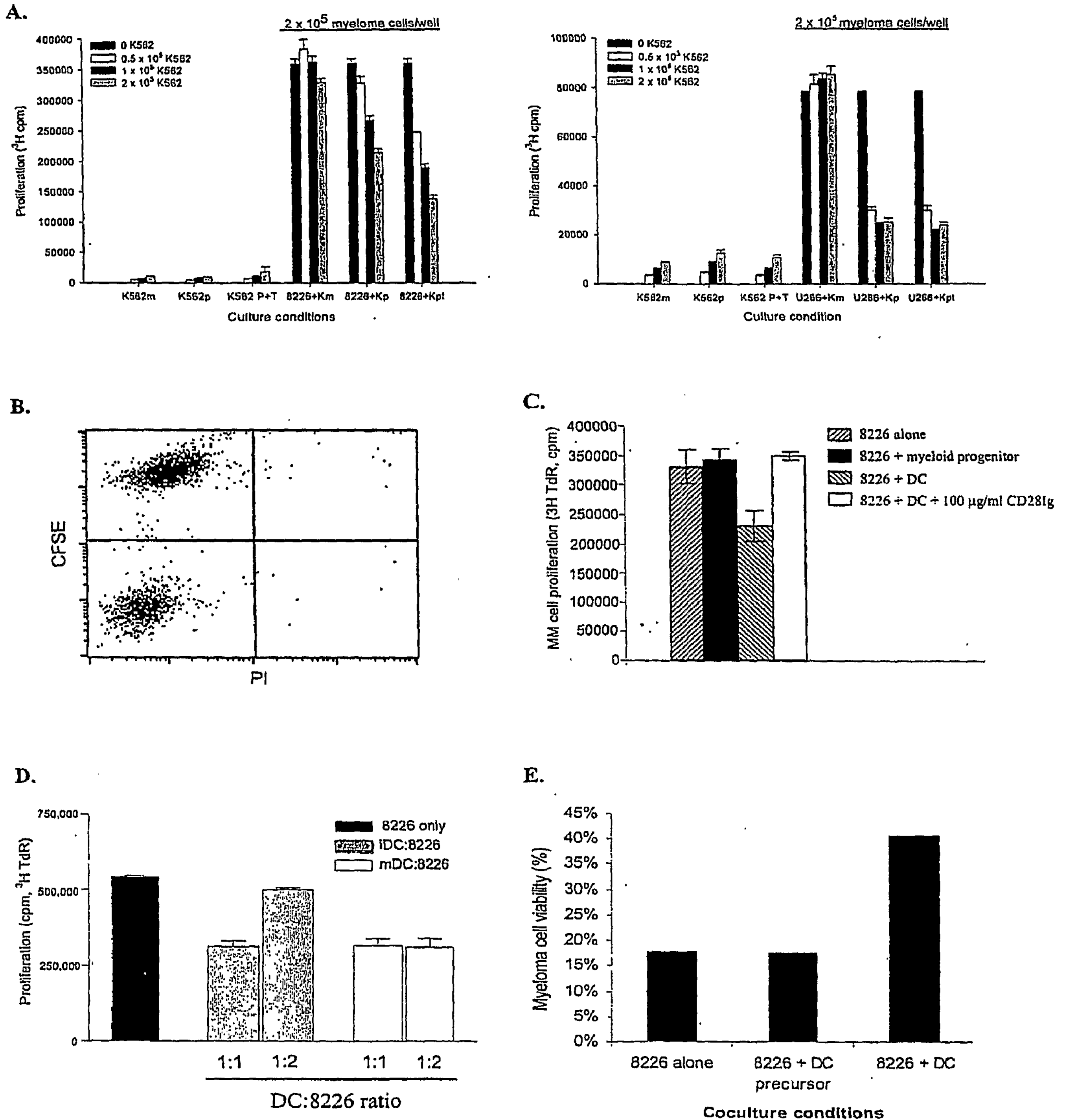


Figure 7

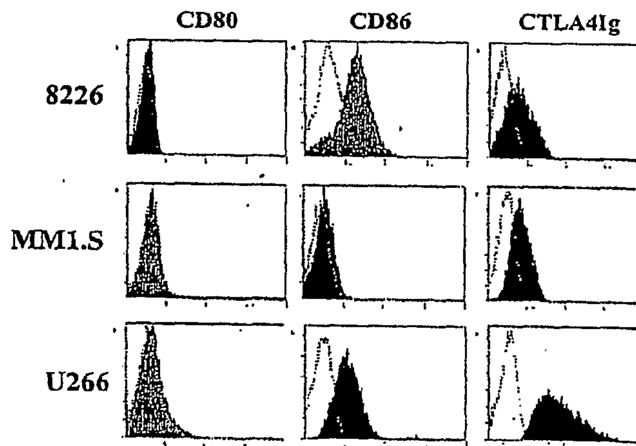


Figure 8

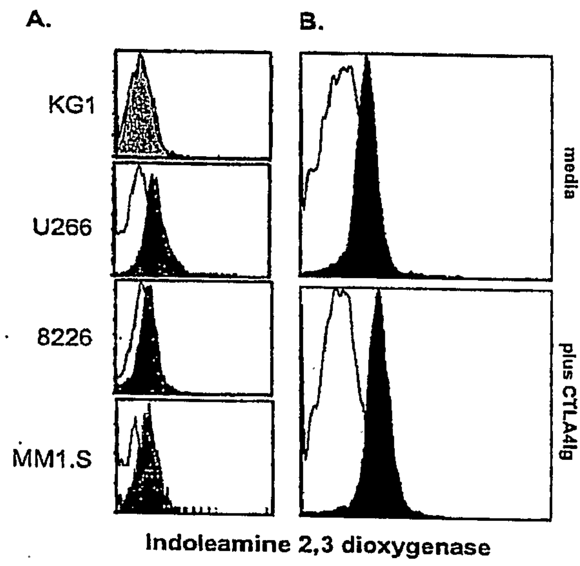


Figure 9