Immunosurveillance and therapy of multiple myeloma are CD226 dependent

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Multiple myeloma (MM) is an age-dependent hematological malignancy. Evaluation of immune interactions that drive MM relies on in vitro experiments that do not reflect the complex cellular stroma involved in MM pathogenesis. Here we used Vk*MYC transgenic mice, which spontaneously develop MM, and demonstrated that the immune system plays a critical role in the control of MM progression and the response to treatment. We monitored Vk*MYC mice that had been crossed with Cd226 mutant mice over a period of 3 years and found that CD226 limits spontaneous MM development. The CD226-dependent anti-myeloma immune response against transplanted Vk*MYC MM cells was mediated both by NK and CD8+ T cells through perforin and IFN-γ pathways. Moreover, CD226 expression was required for optimal antitumor efficacy of cyclophosphamide (CTX) and bortezomib (Btz), which are both standardly used to manage MM in patients. Activation of costimulatory receptor CD137 with mAb (4-1BB) exerted strong antitumor activity, while inhibition of coinhibitory receptors PD-1 and CTLA-4 had no effect. Taken together, the results of this study provide in vivo evidence that CD226 is important for MM immunosurveillance and indicate that specific immune components should be targeted for optimal MM treatment efficacy. As progressive immunosuppression associates with MM development, strategies aimed to increase immune functions may have important therapeutic implications in MM.

Introduction

Multiple myeloma (MM) is the second most common hematological malignancy in the world (1). Despite recent progress in new therapeutic options, this disease remains incurable, with a median survival not exceeding 5 years in adults. MM is characterized by the proliferation of clonal, long-lived plasma cells (PCs) within the BM associated with bone destruction, serum monoclonal gammopathy, and organ dysfunction (1). MM often evolves at a slow rate from an age-dependent premalignant stage called monoclonal gammopathy of undetermined significance (MGUS) (2). The driving forces that promote the evolution from MGUS to active MM have remained obscure until now. Although a number of oncogenic events are associated with MM evolution (2, 3), the pathogenesis of MM could reflect not only cell-intrinsic alterations, but also the impact of host immune reactions. Indeed, accumulating evidence over the past decades demonstrates that the immune system plays a pivotal role in the surveillance of cancer (4, 5). Both innate and adaptive immune cells can recognize and destroy malignant PCs (6–9), and progressive immune suppression has been involved in the progression of MM (10, 11). Additional proof of MM control by immune components derives from the clinical use of immunomodulatory drugs (IMiDs) whose antitumor properties rely, at least partially, on the costimulation of T and NK cells (12–14).

The interaction between CD226 (DNAM-1) with its ligands CD155 (PVR, necl-5) and CD112 (nectin 2) has recently emerged as a critical mechanism of immune defence against a wide range of malignancies (15–18). CD226 was originally described as an adhesion coreceptor stimulating NK cell- and CD8+ T cell-mediated cytotoxicity against tumor cells (19). It has now become clear that this receptor has broader implications than originally thought in controlling NK cell and T cell function (20, 21). CD226 has been implicated in immunological synapse formation (22), T cell proliferation and differentiation (23), and cytokine secretion (21, 24). Interestingly, CD112 and CD155 are often present at the surface of malignant PCs in MM patients, and CD226 strongly contributes to NK cell–mediated killing of MM cells in vitro (8, 9). Moreover, therapeutic agents commonly used in the management of MM, such as melphalan and bortezomib (Btz), upregulate CD226 ligands and sensitize MM cells to NK cell–mediated cytotoxicity (25, 26). Altogether, this suggests that CD226 may play an important role in MM pathogenesis, and modulation of this pathway may have therapeutic implications.
Most of our understanding of immune interactions with MM relies on in vitro experiments. Given the importance of BM stroma in the biology of MM, and the complex cellular and cytokine interactions contributing to MM development, the previous results should be taken with some caution (27). Vk*MYC transgenic mice, which drive sporadic MYC oncogene expression in germinal center B cells, have recently emerged as the most relevant mouse model of MM currently available (28, 29). Vk*MYC mice spontaneously develop MGUS that progresses into indolent MM over 20–50 weeks, and closely mirrors human MM pathological progression. This includes PC neoplasia restricted to the BM, bone destruction, and monoclonal gammapathy (28).Vk*MYC mice were shown to respond to most of the conventional antmyeloma drugs and thus should be taken with some caution (27). Vk*MYC transgenic mice, which drive sporadic MYC oncogene expression in germinal center

elements, have recently emerged as the most relevant mouse model of MM currently available (28, 29). By contrast, it demonstrated that CD226 significantly limited tumor burden in Vk*MYC mice (Figure 1E and Supplemental Figure 2). By contrast, this receptor was only expressed on a small fraction of PCs from normal mice (Supplemental Figure 1). Previous studies suggested that CD226 contributes to shape tumor immunogenicity, a process called cancer immunoediting (4), and that a decrease in CD155 expression on tumor cells may contribute to tumor immune escape (18). However, we observed that the level of CD155 expression on B220 CD138+ malignant PCs was similar between WT and Cd226−/− Vk*MYC mice, demonstrating that although CD226 impacts tumor development and survival, there is no detectable immunoediting of malignant PCs through Cd226/Cd155 interactions (Figure 1F).

**CD226 interaction with CD155 on PCs limits MM development.** We next investigated the mechanisms involved in the control of MM through the CD226 receptor. Given the timeframes required to modify immune components in the spontaneous Vk*MYC model, we decided to use a tractable transplantable model of Vk*MYC MM (Supplemental Figure 2). First, to confirm the role of CD226 receptor in the control of MM growth, WT and Cd226−/− mice were injected with 2 × 10^6 Vk12653 MM cells and were bled every 2 weeks to analyze M-spike levels. We observed a striking increase in serum paraprotein levels between Cd226−/− and WT mice 3 weeks after injection (Figure 2A). After 5 weeks, the number and the percentage of B220 CD138+ PCs recovered from the spleen and the BM were both significantly increased in Cd226−/− mice as compared to WT mice (Figure 2, B and C, and Supplemental Figure 3). Similar results were obtained with an independent MM cell line derived from Vk*MYC transgenic mice, Vkt12598 (Figure 2D and Supplemental Figure 2). Finally, we found that mice injected with Vkt12598 cells succumbed significantly faster to MM in the absence of CD226 (Figure 2E). Decreased survival of Cd226−/− mice compared to WT mice was also observed following the injection of low numbers of Vk12653 cells (Supplemental Figure 3). Given the high level of CD155 expression on Vk*MYC cells, we wanted to determine whether CD155 was involved in the CD226 control of Vk*MYC MM cells. To this end, we transplanted Vk*MYC cells into WT mice injected with anti-CD155, anti-CD226 or control Ig (clg) as described in Methods. Both anti-CD155 mAb- and anti-CD226 mAb-treated mice had higher γ-globulin levels 3 weeks after injection as compared to clg-treated mice (Figure 2F). The percentages of B220 CD138+ PCs in the spleen and the BM were also significantly increased when CD155 or CD226 receptors were blocked (Figure 2G). CD155 can be expressed on several immune cell types after activation, including DC, macrophages, and T lymphocytes. To determine whether endogenous or tumor-derived CD155 was important for the control of MM progression through CD226, we monitored MM growth in Cd155−/−, Cd155+/+, and Cd155−/− littermates treated with clg or anti-CD155. We did not observe any significant differences in MM growth between Cd155−/−, Cd155−/−, and Cd155+/− mice (Figure 2H). By contrast, anti-CD155 blockade increased tumor burden in Cd155+/− mice, suggesting that
NK cells and CD8+ T cell–tumor immunosurveillance (32, 33). Therefore, to gain more insight into the mechanism of CD226-dependent control of MM by NK cells and CD8+ T cells, we analyzed MM burden in mice depleted of NK cells and/or CD8+ T cells using mAbs. Three weeks after injection of Vk12653 cells, both NK cell–depleted mice (anti-asialoGM1 [anti-AsGM1]) and CD8+ T cell–depleted mice (anti-CD8β) had higher paraprotein levels than cIg-treated mice (Figure 3A). Interestingly, mice that lacked both NK cells and CD8+ T cells had significantly higher γ-globulin levels and spleen weight than mice that lacked only one of these two lymphocyte subsets (Figure 3A and Supplemental Figure 4) suggesting that NK cells and CD8+ T cells play additive roles in MM control in vivo.

The granule exocytosis pathway involving perforin and granzymes, and the release of proinflammatory cytokines such as IFN-γ, are the two main mechanisms contributing to NK cell– and CD8+ T cell–tumor immunosurveillance (32, 33). Therefore, to gain more insight into the mechanism of CD226-dependent control of MM by NK cells and CD8+ T cells, we analyzed MM burden in the spleen and the BM of NK cell– or CD8+ T cell–depleted mice. Additionally, these results demonstrate a key role for CD226 interaction with CD155 in controlling MM progression. Altogether, these observations provide evidence for an important role of NK cells and CD8+ T cells in the control of MM development.

NK cells and CD8+ T cells exert antimyeloma activity through perforin and IFN-γ pathways. To characterize the immune cells involved in the CD226-dependent antimyeloma response in vivo, we first monitored MM burden in mice depleted of NK cells and/or CD8+ T cells using mAbs. Three weeks after injection of Vk12653 cells, both NK cell–depleted mice (anti-asialoGM1 [anti-AsGM1]) and CD8+ T cell–depleted mice (anti-CD8β) had higher paraprotein levels than cIg-treated mice (Figure 3A and Supplemental Figure 4). The percentage of B220+CD138+ PCs recovered from tumor CD155 is more important than endogenous CD155 in controlling MM progression. Altogether, these results demonstrate a key role for CD226 interaction with CD155 in the control of MM development.

Figure 1. CD226 limits spontaneous MM progression. (A–C) C57BL/6 Cd226+/+ (n = 55), Cd226–/– (n = 27), and Cd226–/– Vk*MYC transgenic mice, along with Cd226–/– (n = 12), Cd226–/– (n = 15), and Cd226–/– (n = 9) nontransgenic littermate controls, were monitored for MM development (A and B) and survival (C). (A and B) Cd226+ Vk*MYC mice have higher γ-globulin levels. (A) Graph showing the mean γ-globulin levels ± SEM in the serum of the indicated strains of mice over time. (B) Representative SPEP of Cd226+/+ and Cd226–/– Vk*MYC transgenic mice after 500 days. Arrows indicate M-spike. (C) Kaplan-Meier curves showing a significant decrease in the survival of Cd226–/– Vk*MYC mice as compared to WT and Cd226+/+ Vk*MYC littermates. (D–F) Cd226+ Vk*MYC transgenic mice have an increased frequency of malignant PCs expressing CD155 in the BM. The percentage and the number of malignant PCs and their level of CD155 expression were analyzed by FACS in the BM of Cd226+/+ (n = 15) and Cd226–/– (n = 14) Vk*MYC transgenic mice and in Cd226–/– (n = 6) and Cd226–/– (n = 10) nontransgenic littermate controls. (D and E) Graphs (D) and representative FACS plots (E) showing the mean frequency and number ± SEM of CD138+CD155+ malignant PCs in the BM of the indicated strains of mice at 450 days of age. (F) Graph showing similar level of CD155 expression on B220+CD138+ BM PCs from Cd226+/+ Vk*MYC and Cd226–/– Vk*MYC mice. Each symbol represents 1 individual mouse. *P < 0.05, **P < 0.01, ***P < 0.001; Mann-Whitney U test (A and D) Mantel-Cox test (C).
M-spike levels were significantly higher in Ifng−/− mice (Figure 3B), suggesting that perforin and IFN-γ both participate in the antmyeloma immune response, but at slightly different times. Perforin and IFN-γ may also have slightly different roles depending on the location of the MM. Indeed, we found that the antimyeloma effects of perforin were restricted to the BM, where Pfp−/− mice contained mice deficient of perforin (Pfp−/−) and IFN-γ (Ifng−/−). Within 1 week after Vk12653 MM cell injection, we detected higher paraprotein levels in Pfp−/− mice than in Ifng−/− and WT mice (Figure 3B). After 3 weeks, both Pfp−/− and Ifng−/− mice had higher paraprotein levels than WT mice (Figure 3B). Finally, while no significant differences were observed after 5 weeks between WT and Pfp−/− mice, the M-spike levels were significantly higher in Ifng−/− mice (Figure 3B), suggesting that perforin and IFN-γ both participate in the antmyeloma immune response, but at slightly different times. Perforin and IFN-γ may also have slightly different roles depending on the location of the MM. Indeed, we found that the antmyeloma effects of perforin were restricted to the BM, where Pfp−/− mice contained
analyzed by flow cytometry. We observed a significant decrease in CD8+ T cell and NK cell percentages in the blood of Vk*MYC mice as compared to nontransgenic mice (Figure 4, A and B). A similar decrease in NK cells and CD8+ T cells was observed in the BM of mice injected with Vk12653 MM cells, as compared to noninjected mice (Figure 4C). Finally, we found an inverse correlation between the number of BM PCs and the percentage of NK and CD8+ T cells, demonstrating that MM burden is associated with a decrease in the percentage of NK and CD8+ T cells in the BM (Figure 4D).

We next analyzed CD226 expression on CD8+ T cells and NK cells and observed that, in both spontaneous and transplanted Vk*MYC models, a decrease in cell surface expression of CD226 on CD8+ T cells and NK cells was associated with MM progression (Figure 4, E and F). In addition, the number of BM PCs was inversely correlated to the expression of CD226 on NK and CD8+ T cells (Figure 4G). Altogether, our results show that progressive immune defects are associated with MM development.

By contrast, Ifng−/− mice had higher spleen weight and contained significantly higher percentages and numbers of MM cells in the spleen than WT and Pfp−/− mice (Figure 3D and Supplemental Figure 4). Finally, we found that IFN-γ-deficient mice injected with Vk12653 or Vk12598 MM cells had a significantly shorter survival than WT or Pfp−/− mice (Figure 3, E and F). Therefore, although perforin and IFN-γ are both involved in the immune control of MM, the impact of IFN-γ deficiency was more pronounced, reinforcing the importance of this pathway in disease control by immune cells.

Progressive immune defects are associated with MM progression in vivo. MM has been associated with a progressive dysfunction of NK cells and CD8+ T cells (10, 11). To better understand the impact of myeloma progression on host immune responses, we first analyzed immune populations during MM development in vivo. Vk*MYC mice bearing spontaneous MM and nontransgenic control mice were bled at 600 and 700 days, and peripheral blood was analyzed by flow cytometry. We observed a significant decrease in CD8+ T cell and NK cell percentages in the blood of Vk*MYC mice as compared to nontransgenic mice (Figure 4, A and B). A similar decrease in NK cells and CD8+ T cells was observed in the BM of mice injected with Vk12653 MM cells, as compared to noninjected mice (Figure 4C). Finally, we found an inverse correlation between the number of BM PCs and the percentage of NK and CD8+ T cells, demonstrating that MM burden is associated with a decrease in the percentage of NK and CD8+ T cells in the BM (Figure 4D). We next analyzed CD226 expression on CD8+ T cells and NK cells and observed that, in both spontaneous and transplanted Vk*MYC models, a decrease in cell surface expression of CD226 on CD8+ T cells and NK cells was associated with MM progression (Figure 4, E and F). In addition, the number of BM PCs was inversely correlated to the expression of CD226 on NK and CD8+ T cells (Figure 4G). Altogether, our results show that progressive immune defects are associated with MM development.
Optimal antmyeloma therapy requires CD226. It is now well established that the in vivo antitumor efficacy of chemotherapy can rely on the activation of the immune system (34, 35). However, in the absence of a mouse myeloma model, the role of immune components in the clinical efficacy of antmyeloma drugs such as Btz and cyclophosphamide (CTX) remained to be determined.

To address this question, cohorts of WT and Cd226–/– mice were injected with Vk12653 MM cells. After 3 weeks, when mice started to develop measurable M-spike, mice were injected either with cIg or anti-CD226 mAbs and were subsequently treated with PBS, Btz (i.p.; 0.5 mg/kg), or CTX (i.p.; 50 mg/kg) twice a week (Supplemental Figure 5). Evolution of tumor burden was assessed every week by

Figure 4. Progressive immunosuppression is associated with myeloma progression in vivo. (A and B) Representative FACS plots (A) and graphs (B) showing the percentages of TCRβ+CD8+ cells and NK1.1+ TCRβ– NK cells in the peripheral blood of Cd226–/– Vk*MYC mice (n = 20) and nontransgenic control mice (n = 7) at 600 and 700 days. (C) Graphs showing the numbers of TCRβ+CD8+ cells and NK1.1+ TCRβ– NK cells in the BM of naive mice or mice injected with Vk12653 MM cells for 5 weeks. Representative experiment out of 2 involving groups of n = 5–10 mice. (D) Graphs showing the correlation between the percentage of NK and CD8+ T cells and the number of BM PCs in the BM of mice injected with Vk12653 cells for 5 weeks (n = 61). Pearson r coefficient and associated P values are shown. (E) Graphs showing the mean fluorescence intensity (MFI) of CD226 on CD8+ T cells and NK cells as in A. (F) Representative histogram plots and graphs showing the MFI of CD226 staining on NK cells and CD8+ T cells as in C. Isotype control staining (gray line), and CD226 staining on naive mice (black line) and MM-bearing mice (dotted line) are shown. (G) Graph showing the correlation between the expression of CD226 on NK cells and CD8+ T cells and the number of BM PCs in the BM of mice injected with Vk12653 cells for 5 weeks (n = 20). Pearson r coefficient and associated P values are shown. Each symbol represents 1 individual mouse. *P < 0.05, **P < 0.01, ***P < 0.001; paired Student’s t test (B and E) and Mann-Whitney U test (C and F).
SPEP (Figure 5, A and B). CTX was very effective in limiting tumor progression. All groups of mice (WT mice plus Ig, WT mice plus anti-CD226, or Cd226–/– mice) had almost undetectable M-spike after 3 weeks of treatment, while vehicle-treated mice all developed severe paraproteinemia (Figure 5, A and B). Vk12653 cells have been selected for increased resistance to Btz treatment (30, 31). Indeed, we found that Vk12653 cells were less sensitive to Btz than CTX, but we still observed a significant reduction in γ-globulin levels in WT mice treated with Btz (Figure 5, A and B). Surprisingly, in Cd226–/– mice or WT mice injected with anti-CD226 mAbs, Btz treatment failed to limit M-spike progression, suggesting that the absence of CD226 in both the BM and the spleen (Figure 5, C and D, and Supplemental Figure 5). As a consequence, CTX-treated Cd226–/– mice quickly succumbed to MM after the end of the treatment, unlike CTX-treated WT mice that survived significantly longer than vehicle-treated mice (Supplemental Figure 5). Btz therapy also induced a significant decrease in tumor burden in WT mice, although it was less effective in limiting MM progression than CTX (Figure 5, C and D, and Supplemental Figure 5). Surprisingly, the percentages and the numbers of CD138+CD155+ tumors in the BM and spleen of Cd226–/– mice or anti-CD226–injected WT mice were not significantly reduced after Btz treatment, suggesting that the in vivo efficacy of this agent requires an intact CD226 pathway (Figure 5, C and D, and Supplemental Figure 5). To confirm these findings, we performed similar experiments using another Vk*MYC cell line. While Btz treatment significantly increased the survival of WT mice injected with Vk12598 MM cells, we observed that its therapeutic effect was compromised in Cd226–/– mice (Figure 5E). Altogether, these results demonstrate that the immune system affects the in vivo efficacy of the antimyeloma drugs CTX and Btz.

**Therapeutic efficacy of anti-CD137 immunotherapy against myeloma.** Harnessing the immune system to fight tumors has recently proven to be a successful strategy to treat cancer patients (36). mAbs blocking lymphocyte inhibitory receptors programmed death 1 (PD-1;
CD279) and CTLA-4 (cytotoxic T lymphocyte antigen 4; CD152) or stimulating coreceptor CD137 (4-1BB, TNFRSF9) are among the most promising therapies currently in the clinic (37–39). However, due to the lack of reliable MM models, the efficacy of such therapies remain unclear in MM. To answer such questions, WT mice were prophylactically injected with clg, anti-CD137, anti–PD-1, or anti–CTLA-4 and subsequently challenged with 2 × 10^6 Vk12653 MM cells. Representative FACs plot showing the percentages (A) and graphs showing the numbers (B) of CD138^+CD155^+ PCs in the BM at the end of the treatment. Data are representative of 2 independent experiments involving groups of n = 10 mice. (C–F) WT mice were injected i.v. with 2 × 10^6 Vk12653 MM cells. After 3 weeks, mice were injected either with IgG or anti-CD137 mAbs. Representative FACs plot and graph showing the percentages (C), the mean γ-globulin percentages (D), the mean numbers ± SEM of malignant CD138^+CD155^+ PCs in the BM (E) and the survival (F) of the indicated groups of mice. Each symbol represents 1 individual mouse. (G) WT mice were injected i.v. with 4 × 10^6 Vk12598 MM cells. After 1 week, mice were treated with IgG or anti-CD137 mAb twice a week for 4 weeks, and their survival was monitored. Experiment involving groups of n = 10 mice. (H) WT and Cd226^−/− mice were injected i.v. with 2 × 10^6 Vk12653 MM cells. After 3 weeks, mice were injected either with IgG or anti-CD226 mAb and were subsequently treated with clg or anti-CD137. Graphs show the percentages and the mean numbers ± SEM of malignant CD138^+CD155^+ PCs in the BM at the end of the treatment. Data are pooled from 2 independent experiments. **P < 0.01, ***P < 0.001, ****P < 0.0001; Mann-Whitney U test (B, D, E, and H) and Mantel-Cox test (F and G).

Figure 6. Therapeutic efficacy of anti-CD137 immunotherapy against myeloma. (A and B) WT mice were injected with clg, anti-CD137, anti–PD-1, or anti–CTLA-4 and subsequently challenged with 2 × 10^6 Vk12653 MM cells. Representative FACs plot showing the percentages (A) and graphs showing the numbers (B) of CD138^+CD155^+ PCs in the BM at the end of the treatment. Data are representative of 2 independent experiments involving groups of n = 10 mice. (C–F) WT mice were injected i.v. with 2 × 10^6 Vk12653 MM cells. After 3 weeks, mice were injected either with IgG or anti-CD137 mAbs. Representative FACs plot and graph showing the percentages (C), the mean γ-globulin percentages (D), the mean numbers ± SEM of malignant CD138^+CD155^+ PCs in the BM (E) and the survival (F) of the indicated groups of mice. Each symbol represents 1 individual mouse. (G) WT mice were injected i.v. with 4 × 10^6 Vk12598 MM cells. After 1 week, mice were treated with IgG or anti-CD137 mAb twice a week for 4 weeks, and their survival was monitored. Experiment involving groups of n = 10 mice. (H) WT and Cd226^−/− mice were injected i.v. with 2 × 10^6 Vk12653 MM cells. After 3 weeks, mice were injected either with IgG or anti-CD226 mAb and were subsequently treated with clg or anti-CD137. Graphs show the percentages and the mean numbers ± SEM of malignant CD138^+CD155^+ PCs in the BM at the end of the treatment. Data are pooled from 2 independent experiments. **P < 0.01, ***P < 0.001, ****P < 0.0001; Mann-Whitney U test (B, D, E, and H) and Mantel-Cox test (F and G).

CD279) and CTLA-4 (cytotoxic T lymphocyte antigen 4; CD152) or stimulating coreceptor CD137 (4-1BB, TNFRSF9) are among the most promising therapies currently in the clinic (37–39). However, due to the lack of reliable MM models, the efficacy of such therapies remain unclear in MM. To answer such questions, WT mice were prophylactically injected with clg, anti-CD137, anti–PD-1, or anti–CTLA-4, and subsequently challenged with Vk^*MYC MM cells. We could not detect any significant differences in tumor burden between control mice treated with anti–PD-1, anti–CTLA-4, and clg (Figure 6, A and B, and Supplemental Figure 6). By contrast, a striking reduction in the number and percentages of malignant PCs were observed in mice treated with anti-CD137 mAbs (Figure 6, A and B, and Supplemental Figure 6). We next analyzed the antmyeloma effect of anti-CD137 mAbs as a therapeutic agent. Cohorts of WT mice were injected with Vk12653 MM cells. After 3 weeks, when mice started to develop measurable M-spike, mice were injected either with clg or anti-CD137 mAbs. Analysis of serum Ig levels and PC content in the BM and the spleen after 3 weeks of treatment revealed that anti-CD137 mAbs induced a drastic reduction in myeloma burden in most of the mice treated (Figure 6, C–E). Long-term survival analyses revealed that anti-CD137 mAb–treated mice have a significantly longer survival than PBS-treated mice (Figure 6F). These findings were confirmed in another in vivo model of MM, since we observed that anti-CD137 treatment also significantly increased the survival of WT mice injected with Vk12598 MM cells (Figure 6G). Surprisingly, anti-CD137 treatment also led to a significant reduction of MM development in Cd226^−/− mice or in mice treated with anti-CD226 mAbs (Figure 6H and Supplemental Figure 6), suggesting that the presence of functional CD226 is dispensable for the antimyeloma response to anti-CD137 mAb immunotherapy.

Anti-CD137 immunotherapy against myeloma requires NK and CD8^+ T cells. To gain more insight into the mechanism involved in the antmyeloma effect of anti-CD137 mAb, we performed a phenotypic analysis of immune cells infiltrating the BM of Ig-treated
CD137 therapy, while their percentage among BM CD4+ T cells was significantly decreased (Figure 7, A–C, and Supplemental Figure 7). As a consequence, anti-CD137 therapy induced a strong increase in the ratio between CD8+ T cells and Tregs. Recent evidence suggests that mAb-based immunotherapy can act through the Fc- or anti-CD137 mAb–treated mice. We observed a robust increase in the number and the percentages of CD8+ T cells infiltrating the BM after 3 weeks of anti-CD137 mAb treatment, as compared to the cIg-treated mice (Figure 7, A–C, and Supplemental Figure 7). By contrast, the number of BM FOXP3+ Tregs remained stable after anti-CD137 therapy, while their percentage among BM CD4+ T cells was significantly decreased (Figure 7, A–C, and Supplemental Figure 7). As a consequence, anti-CD137 therapy induced a strong increase in the ratio between CD8+ T cells and Tregs. Recent evidence suggests that mAb-based immunotherapy can act through the Fc-
dependent depletion of tumor-associated Tregs (40). We found that a significant fraction of Tregs express CD137 (Supplemental Figure 7), suggesting that a depletion of Tregs by anti-CD137 mAb may be involved in its therapeutic effects. However, Fc receptors were not involved in the antitumor effects of anti-CD137 mAbs (of the IgG2a isotype) since CD8+ T cell expansion, as well as BM MM cell burden, was similar in WT mice and mice lacking the Fc receptor common γ-chain (Fcer1g−/−) or the low affinity receptor for IgG2a/IgG1/IgG2b (Fggr3a−/−) (Figure 7; D and E). These results suggested that anti-CD137 mAbs may directly act on effector cells. Therefore, we analyzed NK and CD8+ T cell activation early after the beginning of the treatment and found that the CD69 activation marker was increased on BM NK cells and CD8+ T cells 6 days after CD137 mAb injection (Figure 7, F and G). By contrast, neither NK cells nor CD8+ T cells showed signs of activation after anti-PD-1 therapy, and the CD8+ T cell activation after anti-CD137 mAb treatment was similar to NK cells and CD8+ T cells 6 days after CD137 mAb injection (Figure 7, F and G). We then treated MM-bearing mice with depleting mAbs against CD8+ T cells in anti-CD137–mediated antimyeloma activity. Altogether, our results demonstrated that standard drugs used in the management of MM, CTX, and Btz also required CD226 for optimal antimyeloma efficacy in vivo. This seminal work suggests that CD226 may play an important role in MM pathogenesis. The translation of such findings in human MM patients and a better comprehension of immune receptor ligands that control the efficacy of antimyeloma therapy may have strong clinical applications.

Discussion

Until now, the lack of reliable mouse models has limited the understanding of immune interactions with MM in vivo. In this study — using the most clinically relevant mouse model of MM, Vk*MYC transgenic mice — we demonstrated that the immune system limits spontaneous MM burden in vivo. We provided strong evidence that immune control of MM is mediated by NK cells and CD8+ T cells through CD226 interaction, with CD155 expressed on the cell surface of transformed PCs. Moreover, our results demonstrated that standard drugs used in the management of MM, CTX, and Btz also required CD226 for optimal antimyeloma efficacy in vivo. This seminal work suggests that CD226 may play an important role in MM pathogenesis. The translation of such findings in human MM patients and a better comprehension of immune receptor ligands that control the efficacy of antimyeloma therapy may have strong clinical applications.

Although a number of oncogenic events are associated with myeloma evolution, the driving forces that promote the evolution from MGUS to smoldering and active myeloma have remained obscure (2, 41). Primary oncogenic events such as IgH translocations and hyperdiploidy have been associated with cell cycle deregulation, PC survival, and MGUS, while secondary IgH translocations, RAS mutations, and overexpression of MYC and MYC target genes have been associated with progression to active myeloma (2, 42). Our study suggests that host immune reaction may represent another key factor involved in the progression of MM. Management of MGUS and smoldering MM mainly involves a “watch and wait” strategy. Our results suggest that immune monitoring and immunotherapy may greatly impact the outcome for patients with a high risk of developing MM.

Oncogene activation is a powerful inducer of cellular stress that not only activates apoptosis or senescence cellular programs (43), but also stimulates immune recognition through the upregulation of NKp46 and CD226 ligands (25, 44–46). It has recently been demonstrated in the EµMYC transgenic mouse model that replication stress induced by MYC overexpression is a strong inducer of CD155 expression (47). Our study demonstrates that malignant PCs developing in Vk*MYC mice all displayed a high expression of CD155, as compared to normal PCs. Although no formal proof has been established, this suggests that genomic stress associated with MYC overexpression may be at the origin of CD155 expression on transformed PCs in Vk*MYC mice. Similar mechanisms may occur in MM patients, since MYC and MYC target genes — as well as CD155 — are often overexpressed in malignant PCs isolated from patients (8, 28). Our study suggests that the expression of CD155 may represent a strong determinant of MM immunogenicity, and an extensive understanding of the clinical impact of this ligand expression in MM patients is now required. CD226 was also shown to interact with CD112 (48, 49), but the relevance of CD112/CD226 interactions in mice remains unclear (50). Although, CD112 expression was usually low or absent on Vk*MYC MM cells, this ligand was found to be expressed on human MM cells and could also play a role in the pathogenesis of this disease (8).

Recent studies indicate that the efficacy of several chemotherapies in vivo relies on the immune system (34). Although most antitumor agents are thought to act primarily through direct cytotoxic effects, additional effects on the immune system may contribute to their efficacy in vivo. We observed that the absence of CD226 or neutralization of this receptor affected the antitumor activity of CTX and Btz, providing strong evidence that immune components and CD226 may represent strong determinants of antitumor therapy efficacy. Low-dose CTX has been shown to deplete Tregs and restore T and NK cell functions (51–54). The depletion or neutralization of Tregs and the reactivation of NK and CD8+ effector lymphocytes upon CTX treatment may account for the immune-dependent action of CTX in our experiment.

Despite being one of the most active drugs used in the management of MM, malignant PCs often acquire resistance to Btz, leading to patient relapse (29). Therefore, to mimic more closely what may happen in human patients, we chose to identify the role of immune components using Btz-resistant tumor cells, Vk12653 and Vk12598 (30, 31). Although Vk12653 and Vk12598 cells were less sensitive to Btz than CTX, the progression of MM was still significantly affected by Btz treatment in immune-competent mice. However, the antitumor efficacy of Btz was completely lost in the absence of CD226, suggesting that the in vivo efficacy of this drug mainly relies on immunity. Such results suggest that immunotherapeutic approaches may greatly enhance the efficacy of this drug in MM, and future experiments will have to determine the underlying mechanisms of the CD226-dependent effects of Btz. Part of the effect of this drug might be mediated through NK cell activation, since Btz was shown to augment the efficacy of adoptively transferred NK cells in both murine tumor models and cancer patients (55, 56). Indeed, proteasome inhibitors alter MHC class I (MHC-I) peptide processing machinery, and MM cells treated with Btz have reduced cell surface expression of MHC-I and are more susceptible to NK cell–mediated killing (57). Btz has also been shown to stimu-
late the expression of CD155 and NKG2D ligands at the cell surface of MM cells (26). CD155 expression is already high on Vk*MYC MM cells, but we cannot rule out that an increase in CD155 expression on accessory cells may contribute to the CD226-dependent MM reduction in Btz-treated mice. Alternatively, Btz treatment may also trigger immunogenic cell death of MM cells (34) and stimulate the presentation of tumor antigens to cytotoxic T cells. For example, previous study demonstrated that Btz increases the uptake of dying human myeloma cells by dendritic cells (58).

The inclusion of drugs such as IMiDs and Btz has resulted in a significant prolongation of survival of MM patients. However, MM is still an incurable disease with a median survival not exceeding 5 years in adults (1). Immune stimulatory mAbs have recently emerged as a promising strategy and are currently tested in several malignancies, with the hope of further improving patients’ survival (59). Recent clinical trials have demonstrated a significant therapeutic impact of PD-1 inhibition on a variety of solid tumors (37, 60). The broad expression of PD-1/PD-ligand 1 (PD-L1) in the microenvironment of myeloma suggest this pathway may play an important role in the immune evasion by MM cells (61). Several studies demonstrated that PD-1 or PD-L1 blockade increases immune cell reactivity against malignant PCs (62–64). In our model, we could not observe any therapeutic or notable immune stimulatory effect of anti-PD-1 treatment. However, we do not exclude that such a treatment administered with a different schedule or in combination with other immune stimulatory or chemotherapeutic agents may have potential antitumor activity. Indeed, we found that PD-1 expression is very low on spleen or BM effector cells in the early phase of MM development, possibly explaining the low therapeutic activity of this drug in our model. However, we found that PD-1 was highly upregulated on CD8+ T cells and NK cells following anti-CD137 therapy, suggesting that anti–PD-1 mAbs may combine with anti-CD137 therapy.

Anti-CD137 is one of the most interesting immunostimulatory mAbs tested for cancer therapy (65–69). Currently, BMS-663513, a humanized anti-CD137 antibody, is in clinical trials in patients with solid tumors, including melanoma, renal carcinoma, ovarian cancer, and B cell malignancies (70). Our present results demonstrate that anti-CD137 exerts strong antitumor effects, providing a strong rationale for testing this agent in MM. It would now be interesting to analyze the benefit of combining such treatments with classical MM chemotherapy, such as Btz. Although CD226 had a strong impact in the control of MM burden in our model, the antitumor effects provided by anti-CD137 mAb therapy appeared independent of CD226. CD137-mediated signals were shown to increase DC maturation, to reverse CD8 T cell anergy, and to render effector T cells refractory to Treg-mediated suppression (66). Anti-CD137 mAb–mediated T cell activation may therefore bypass the requirement for CD226 costimulatory signals. Indeed, besides its well-known role in cytotoxicity, CD226 has also been shown to play an important role in T cell activation mediated by antigen-presenting cells (16, 22), and more recently, decreased CD226/CD155 interactions were shown to be an important feature of exhausted CD8+ T cells within human tumors (71). With the progression of MM, CD226 is often downregulated on effector cells (8), and therefore finding an immunotherapeutic agent that allows the targeting of malignant PCs independently of the CD226 pathway is a valuable discovery. Our study suggests that anti-CD137 therapy may bypass the potential immune exhaustion associated with MM disease development. Analysis of BM and spleen lymphocyte populations after immunotherapy revealed that anti-CD137 decreased Treg percentages and simultaneously increased CD8+ T cells, leading to a dramatic increase in the CD8/T cell/Treg ratio. Although such findings are reminiscent of the effect of anti–CTLA-4 mAbs in solid tumors (40), unlike anti–CTLA-4, anti-CD137 effects were not mediated through an Fc-dependent depletion of Tregs. Consistent with a previous study (72), we found that anti-CD137 therapy required the presence of both CD8+ T cells or NK cells, while CD4+ T cells were dispensable. Indeed, NK cells and CD8+ T cells were activated early after the beginning of anti-CD137 therapy, and their depletion abrogated the therapeutic effect of anti-CD137. MM growth relies on a complex network of cytokines and cellular interactions (27). Future experiments will have to determine whether therapy-induced changes in the BM microenvironment may alter this network and, by this mechanism, limit MM development. It would now be interesting to analyze the benefit of combining such treatments with classical MM chemotherapy, such as Btz.

Methods
Mice. All mice used in this study were on a C57BL/6 genetic background. C57BL/6 WT mice were purchased from the Walter and Eliza Hall Institute for Medical Research or ARC Animal Resource Centre. Vk*MYC(Tg[Tkcv3-5-MYC]#F6be)(28), Cd226+/– (16), and Fpr–/–, Ifng–/–, and Rag2–/– Il2rg–/– mice have already been described (73). Cd226–/– mice were provided by Yoshimi Takai (Kobe University, Kobe, Japan). Fcrlg–/– and Fcgr3a–/– mice were provided by Eva Orlowski (Burnet Institute, Melbourne, Australia). All mice were bred and maintained at either the Peter MacCallum Cancer Centre or QIMR Berghofer Medical Research Institute and were used between the ages of 6–14 weeks.

Vk*MYC aging experiments. Vk*MYC transgenic mice were crossed with Cd226–/– mice. Cd226+/– (n = 55), Cd226–/– (n = 27), and Cd226+/– (n = 25) Vk*MYC transgenic littermate controls, as well as Cd226+/– (n = 12), Cd226+/– (n = 15), and Cd226+/– (n = 9) nontransgenic littermate controls, were monitored for MM development and survival during a period of 3 years. The presence of monoclonal Ig (M-spike) and the level of γ-globulin in the serum were quantified by SPEP (HYDRASYS, Sebia Hydragel) every 2 months. The content of malignant PCs in the BM was determined in the different groups of mice postmortem (Supplemental Figure 1). A second cohort of Cd226+/+ (n = 15), Cd226–/– (n = 14) Vk*MYC transgenic mice, Cd226+/– (n = 6), and Cd226+/– (n = 10) nontransgenic littermate controls were culled at 450 days of age to analyze by flow cytometry the percentage of B220+ CD138+ PCs in the BM and the spleen.

Vk*MYC transplantable model. Transplantable Vk*MYC MM cells Vkl2653 and Vkl2598 were generated as described previously (30). To remove any potential contaminant cells that may derive from the original host, we performed 2 successive transplants by i.v. injection of 2 × 106 Vkl2653 cells or 4 × 106 Vkl2598 cells into Rag2–/– Il2rg–/– mice. After the second transplant, all Rag2–/– Il2rg–/– mice developed robust M-splkes weeks after injection. Spleen cells from Rag2–/– Il2rg–/– mice containing at least 50% of malignant PCs were frozen and subsequently used as a reliable source of transplantable Vk*MYC cells for all our experiments (Supplemental Figure 2). The presence of monoclonal Ig in the serum was determined every 1, 3, and 5 weeks after MM cells injection by SPEP. The percentage and the number of B220+ CD138+ PCs was analyzed in the BM and the spleen by flow cytome-
Antimyeloma therapy. For prophylactic treatment, WT or Cd226−/− mice were injected with clg, anti-CD137 (200 μg, 3H3; BioxCell), anti-CD8 (200 μg, RMP1-14; BioxCell), or anti-CTLA-4 (200 μg, UC10-4F10; BioxCell) i.p. on days –1 and 0, and every week after MM cell injection. For a therapy experiment, WT and Cd226−/− mice were injected i.v. with 2 × 105 Vk12653 cells. After 3 weeks, when mice started to develop detectable M-spike, mice were randomly assigned into groups that received vehicle (PBS), CTX (i.p. 50 mg/kg, twice a week), Btz (i.p. 0.5 mg/kg, twice a week), anti-CD137 (100 μg, twice a week, 3H3; BioxCell), or anti-CD8 (100 μg, twice a week, RMPI-14; BioxCell). Alternatively, mice injected i.v. with 4 × 105 Vk12598 MM cells were treated with the indicated antimyeloma drugs 1 week after MM cell injection.

Antibody treatments. In vivo antibody depletion and receptor blockade were performed using the following antibodies: control IgG (100 μg, HRPN; BixoCell), anti-CD4 (100 μg, GK1.5; BixoCell), anti-CD8 (100 μg, 53.5.8; BixoCell), anti-AsGM1 (100 μg; Wako), anti-CD226 (200 μg, 480.1), and anti-CD155 (200 μg, 24.24.3). All antibody injections were given i.p. on days –1 and 0, and every week after MM injection. Alternatively, antibody injections were given on days –15 and 0, and every week after the beginning of the antimyeloma therapy.

Flow cytometry. Single-cell suspensions were stained with the following conjugated mAbs: anti-mouse CD3 (145-2C11), TCR-ε (H57-597), CD4 (RM4-5), FOXP3 (FJK-16s), CD8 (53-6.7), NK1.1 (PK136), CD69 (H1.2F3), CD226 (480.1), CD112 (829038), CD155 (4.24.3), B220 (RA3-682), CTLA-4 (UC10-4B9), H-2Db (28-14-8), H2-Kb (AF6-88-5), Rae-1 (clone 186107), PD-1 (1RMP1-30), PD-L1 (10F9G2), PD-L2 (clone 122), CD137L (185S), CD137L (TKS-1), and CD138 (clone 281-2). All mAbs were purchased from R&D Systems, BioLegend, or eBioscience. Flow count beads (BD Biosciences) were added to all sample acquisitions.

Statistical analysis was achieved using GraphPad Prism software. A nonparametric Mann-Whitney test or unpaired t tests were used for single comparisons between groups. Comparisons between paired samples were analyzed with 2-tailed paired t tests. A Mantel-Cox test was used for survival analysis. Correlation analyses between variables were performed using Pearson rank correlation test. A P value less than 0.05 was considered significant (*P < 0.05, **P < 0.01, ***P < 0.001).

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