Macrophages are an abundant component of myeloma microenvironment and protect myeloma cells from chemotherapy drug-induced apoptosis

Yuhuan Zheng,¹* Zhen Cai,¹,²* Siqing Wang,¹* Xiang Zhang,¹ Jianfei Qian,¹ Sungyoul Hong,¹ Haiyan Li,¹ Michael Wang,¹ Jing Yang¹ and Qing Yi¹

¹Department of Lymphoma and Myeloma, Division of Cancer Medicine, Center for Cancer Immunology Research, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA; and ²Department of Hematology, The First Affiliated Hospital, Medical School of Zhejiang University, Zhejiang 31003, China.

*These authors contributed equally to this work.

Correspondence and reprint request: Qing Yi, M.D., Ph.D., Department of Lymphoma and Myeloma, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 0903, Houston, Texas 77030, USA. Phone: 713 563 9065; Fax: 713 563 9241; E-mail: qyi@mdanderson.org

Short title: Macrophages protect myeloma cell apoptosis.

Scientific Section Heading: Lymphoid Neoplasia

Keywords: Multiple myeloma, macrophages, apoptosis, chemotherapy, microenvironment.
Abstract

Multiple myeloma remains an incurable disease. One of the major problems is that myeloma cells develop drug resistance upon interaction with bone marrow stromal cells. In this study we examined the effects of macrophages (Mφ), a type of stromal cells, on myeloma cell survival and response to chemotherapy. We showed that Mφ, in particular tumor-associated Mφ, are a protector of myeloma cells. The protective effect was dependent upon direct contact between Mφ and myeloma cells. Mφ protected both myeloma cell lines and primary myeloma cells from spontaneous and chemotherapy drug-induced apoptosis by attenuating the activation and cleavage of caspase-dependent apoptotic signaling. These findings are clinically relevant, because we found that CD68+ Mφ heavily infiltrate the bone marrow of patients with myeloma but not the bone marrow of control patients. Thus, our results indicate that Mφ may contribute to myeloma cell survival and resistance to chemotherapeutic treatment in vivo.
Introduction

Multiple myeloma (MM) is a malignant B-cell tumor characterized by proliferation of monoclonal plasma cells in the bone marrow\(^1\). Although chemotherapy is the most effective treatment for MM at present time, myeloma cells often fail to respond to the drugs. Studies have shown that the response of myeloma cells to cytotoxic chemotherapeutics can be attenuated by the presence of bone marrow stromal cells\(^2,3\). However, the mechanisms of myeloma cell proliferation and failure to respond to chemotherapeutic drugs are not fully defined. To better understand the role of different stromal cell components in the bone marrow microenvironment, we examined the effects of macrophages (M\(\phi\)) on myeloma cell survival and response to chemotherapy in this study.

Materials and Methods

Myeloma cells, antibody, and reagents

Primary myeloma cells were isolated from bone marrow aspirates of myeloma patients. IL-6 and M-CSF antibodies were purchased from R&D Systems (Minneapolis, MN). Melphalan, dexamethasone, cytochalasin D, and FITC-labeled dextran were purchased from Sigma-Aldrich (St. Louis, MO). The study was approved by the Institutional Review Board at The University of Texas M. D. Anderson Cancer Center.

Generation of macrophages

Mononuclear cells from the blood of healthy donors were incubated in 12-well plates for 2 hours (h) at 37°C to remove non-adherent cells. The adherent monocytes were incubated for 7 days in medium with M-CSF\(^4\) to become nM\(\phi\). nM\(\phi\) were cultured for an additional 72 h with tumor-culture conditioning medium (TCCM) of myeloma cells\(^5\) to generate tM\(\phi\). M\(\phi\) were also generated from blood monocytes of patients with MM in a 7-day culture with M-CSF and used in the experiments.

Apoptosis assay
Annexin-V staining was used to detect apoptosis in myeloma cells as described previously\(^6\). To exclude apoptotic macrophages, cultured cells were stained with PE-conjugated anti-CD138 antibody and FITC-conjugated Annexin V, and apoptotic myeloma cells were identified as CD138\(^+\)Annexin V\(^+\) cells.

**Immunohistochemistry analysis**

Sections of bone marrow biopsies from MM and control patients were examined by immunohistochemistry staining as described previously\(^7\).

**Results and Discussion**

To evaluate the effect of M\(\phi\) on myeloma cells, we examined whether M\(\phi\) could protect myeloma cells from chemotherapy drug-induced apoptosis. As shown in Figure 1A, coculture of myeloma cells with M\(\phi\) protected myeloma cells from dexamethasone- and melphalan-induced apoptosis (\(P < 0.01\), by Student \(t\) test). However, tM\(\phi\) were more effective than nM\(\phi\) at protecting myeloma cells from melphalan-induced apoptosis in 4 myeloma cell lines examined (Figure 1B; \(P < 0.05\) and \(P < 0.01\)). Next, we examined whether M\(\phi\)-mediated protection requires cell-cell contact. In the experiments, tM\(\phi\) were cocultured with myeloma cells either in direct contact or separated by Transwell insets. Although direct coculture of tM\(\phi\) with myeloma cells conferred protection against melphalan-induced apoptosis (Figure 1C; \(P < 0.01\)), coculture of tM\(\phi\) with myeloma cells in Transwell inserts nearly abolished the ability of tM\(\phi\) to protect myeloma cells.

Likewise, the addition of culture supernatants of tM\(\phi\) to myeloma cells slightly but insignificantly protected the cells from chemotherapy-induced apoptosis (data not shown). To examine the importance of cell-cell contact, blocking antibody specific for adhesion molecule ICAM-1 was used. Addition of anti-ICAM-1 antibody significantly compromised tM\(\phi\)-mediated protection (Figure 1D). These results indicate that cell-cell contact plays a major role in M\(\phi\)-mediated protection of myeloma cell apoptosis.

Furthermore, we examined whether M\(\phi\) can also support primary myeloma cell survival. Primary myeloma cells freshly isolated from patients with MM undergo apoptosis ex vivo in medium unless they are cocultured with stromal cells\(^8\). As shown in Figure 1E
depicting the representative data obtained from experiments with samples from one out of four patients examined, approximately 50% and 80% of primary myeloma cells were apoptotic 24 h and 48 h, respectively, after isolation, whereas fewer than 20% of the cells were apoptotic when cocultured with tMϕ. We also found that coculture with myeloma patients-derived Mϕ significantly protected myeloma cell apoptosis induced by melphalan (Figure 1F). These results indicate that Mϕ, especially tMϕ, may be a protector of myeloma cell apoptosis. Studies have shown that tMϕ are driven by tumor-derived cytokines to acquire a polarized type-2 phenotype, which differ in terms of receptor expression, effector function, and cytokine and chemokine production9.

To elucidate the mechanism underlying Mϕ-mediated protection in myeloma cells, we examined apoptotic signaling pathways in myeloma cells. By using Western blot analysis, we showed that melphalan treatment activates and induces cleavage of caspase-3 and PARP, and downregulated Bcl-xL in myeloma cells (Figure 2A). Coculture of myeloma cells with tMϕ protected myeloma cells from melphalan-induced apoptosis by inhibiting the activation and cleavage of caspase-3 and PARP, and maintaining the levels of Bcl-xL. No changes in the expression of Bcl-2, Bad, or Bax were observed in myeloma cells treated with melphalan in the presence or absence of tMϕ. These results suggest that Mϕ protect myeloma cells from apoptosis via inhibiting Bcl-xL-dependent caspase activation.

Next we examined whether IL-6, one of the most important cytokines for myeloma growth and survival10, plays a role in Mϕ-mediated protection of myeloma cell apoptosis. As shown in Figure 2B, the level of IL-6 was significantly higher in the supernatant of tMϕ than that of nMϕ or TCCM (P < 0.01). To examine the importance of IL-6 in tMϕ-mediated protection, neutralizing antibody against IL-6 was used. As shown in Figure 2C, addition of anti-IL-6 specific antibody did not affect tMϕ-mediated protection of myeloma cells from melphalan-induced apoptosis, indicating that IL-6 did not contribute to the protective effects of tMϕ. It may be possible that the growth and anti-apoptotic signaling generated by cell-cell contact is stronger than those of IL-6 signaling. Further studies are warranted to elucidate the mechanisms.
To rule out the possibility that Mφ engulfed apoptotic myeloma cells so that fewer apoptotic cells were detected in the cocultures, cytochalasin D was used to inhibit the endocytosis ability of Mφ. Myeloma cells were cultured with tMφ pretreated with or without cytochalasin D in the presence of melphalan for 24 hours, and the number of apoptotic cells was determined after the culture. As shown in Figure 2D, 46% of myeloma cells cultured with melphalan became apoptotic, whereas fewer than 10% myeloma cells cocultured with either untreated or cytochalasin D-pretreated tMφ in the presence of melphalan became apoptotic. By using FITC-conjugated dextran, we show that pretreatment of Mφ with cytochalasin D significantly inhibited the ability of Mφ to engulf FITC-conjugated dextran (data not shown). These results indicate that the reduced numbers of apoptotic myeloma cells in the cocultures were indeed the result of apoptosis protection mediated by tMφ.

To evaluate the clinical relevance of our findings, we examined whether Mφ are present in the bone marrow of myeloma patients. Mφ in the bone marrow samples were identified with antibody against CD68, a glycoprotein expressed only by human Mφ. As shown in Figure 2E by the representative staining from one MM and one control patient out of 4 examined, CD68+ Mφ were scarcely found in the bone marrow biopsies of control patients, whereas in patients with MM, CD68+ Mφ were heavily infiltrated in the bone marrow. The quantitative results of infiltrating CD68+ Mφ are shown in Figure 2F. It is evident that significantly increased numbers of CD68+ Mφ were found in the bone marrow biopsies of MM patients than controls ($P < 0.01$). Taken together, these findings indicate that Mφ may be an abundant and important component of the bone marrow stromal cells and play a critical role in vivo in protecting myeloma cells from chemotherapy-induced apoptosis.

**Acknowledgements**

This work was supported by National Cancer Institute grants (R01 CA96569, R01 CA103978, and CA138402), the Leukemia and Lymphoma Society Translational
Research Grant, Multiple Myeloma Research Foundation, and Commonwealth Foundation for Cancer Research. We thank our Departmental Myeloma Tissue Bank for patient’s samples and Ms. Alison Woo for providing editorial assistance.

**Authorship**

Contribution: YZ, ZC, and SW performed the majority of experiments and contributed to the written paper; XZ, JQ, SH, and HL performed experiments; MW provided patient samples and critical suggestions; JY contributed to conceptual idea and performed experiments; and QY contributed to conceptual idea for the paper, experimental design, and writing and editing of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
References

LEGENDS TO FIGURES

Figure 1. Macrophages protect myeloma cells from apoptosis.

(A) Dot plots showing apoptotic myeloma (MM.1S) cells in cultures or cocultures with normal Mφ (nMφ) or TCCM-treated Mφ (tumor-associated Mφ; tMφ) in the presence or absence of dexamethasone (Dex) or melphalan (Mel). Numbers inside dot plots indicate the percentages of live cells. (B) Apoptotic myeloma cells (percentage of melphalan control, which is 100% of apoptotic cells) in cocultures with either nMφ or tMφ in the presence of melphalan. Four commonly used myeloma cell lines, ARP-1, MM.1S, RPMI-8226, and U266, were tested. (C) Percentages of apoptotic myeloma cells in cocultures without or with tMφ either in direct contact (MM+tMφ) or separated by Transwell inserts (MM+tMφ [Transwell]) in the presence of melphalan. Culture of myeloma cells in medium and coculture of myeloma cells with tMφ served as controls. (D) Effects of anti-ICAM-1 antibody on blocking tMφ-mediated myeloma apoptosis protection. Shown is the percentage of apoptotic myeloma ARP-1 cells pretreated with 10 μg/mL of anti-ICAM-1 antibody or mouse IgG, in (co)cultures with tMφ in the presence of melphalan. Culture of myeloma cells in medium and coculture of myeloma cells with tMφ served as controls. Similar results were obtained with other myeloma cell lines. (E) Percentages of spontaneous apoptotic primary myeloma cells in culture medium only (primary MM) or in cocultures with tMφ (primary MM+tMφ) at 24 h and 48 h after isolation of the myeloma cells. Representative results from experiments with primary myeloma cells from one patient out of 4 examined are shown. (F) Percentage of apoptotic myeloma ARP-1 cells in coculture with Mφ generated from MM patients in the presence of melphalan. Representative results from experiments with Mφ from one patient out of three examined using this and other cell lines are shown. *P < 0.05, and **P< 0.01.

Figure 2. The mechanism of macrophage-mediated anti-apoptosis in myeloma cells.

(A) Western blot analysis showing the protein expression of cleaved PARP (cPARP), cleaved caspase-3 (cCas-3), Bcl-xL, Bcl-2, Bad, and Bax in ARP-1 myeloma cells cultured alone or cocultured with tMφ in the presence of melphalan (5 μM). The level of β-actin served as loading control. Results from one representative experiment out of three
performed with ARP-1 are shown. Similar results were obtained with other myeloma cell lines. (B) Levels of IL-6 in normal medium, TCCM, and in the supernatants of nMφ and tMφ, measured by ELISA. (C) Dot plots showing apoptotic myeloma cells in culture medium (Med), in cocultures with tMφ, and in coculture with tMφ and IL-6 neutralizing antibody (αIL-6) in the presence or absence of melphalan (Mel). Numbers inside dot plots indicate the percentages of live cells. (D) Percentages of melphalan (Mel)-induced, Annexin V-positive apoptotic myeloma (ARP-1) cells in culture medium only (Med) or in cocultures with untreated tMφ or with cytochlasin (CD)-pretreated tMφ (CD-tMφ).

Infiltration of Mφ in the bone marrow of myeloma patients: (E) Immunohistochemistry staining by CD68 antibody to identify Mφ in bone marrow biopsies from a control patient without malignancy and from a randomly selected myeloma patient. Representative results from experiments with bone marrow biopsies from one out of 4 myeloma and 4 control patients examined are shown, and (F) Percentages of infiltrated Mφ in bone marrow biopsies of MM and control patients. Data were derived from the numbers (mean + SD) of CD68+ Mφ in a total of 1000 cells counted in bone marrow biopsies of 4 patients with MM and 4 controls.
Figure 1A-D

Figure 1E-F
Figure 2A-D

Figure 2E-F
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