Macrophages promote aberrant DNA repair in multiple myeloma via the CXCL5/8-CXCR2 axis

Mengmeng Dong;[1,2*] Donghua He,[1*] Jinna Zhang,[1,2*] Haimeng Yan,[1] Haoguang Chen,[1] Enfan Zhang,[1] Yili Feng,[2,3] Jingsong He,[1] Xi Huang,[1] Guoqiao Chen,[2,3] Xiuna Sun,[2,3] Fei Cheng,[4] Huiyao Gu,[1] Huanping Wang,[1] Anyong Xie[2,3] and Zhen Cai[1,5]

[1]Bone Marrow Transplantation Center, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou Zhejiang; [2]Institute of Translational Medicine, Zhejiang University School of Medicine and Zhejiang University Cancer Center, Hangzhou, Zhejiang; [3]Innovation Center for Minimally Invasive Technique and Device, Department of General Surgery, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang; [4]Pathology Department, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang and [5]Institute of Hematology, Zhejiang University, Hangzhou, Zhejiang, China

MD, DH and JZ contributed equally as first authors.

Correspondence: Z. Cai
caiz@zju.edu.cn.

A. Xie
anyongxie@zju.edu.cn

Received: January 7, 2025.
Accepted: June 18, 2025.
Early view: June 26, 2025.

https://doi.org/10.3324/haematol.2025.287312

Abstract

Multiple myeloma (MM) is closely associated with abnormal DNA repair and genome instability. The bone marrow microenvironment, particularly myeloma-associated macrophages (MΦ), is critical to the progression of MM. However, there is limited understanding on the role of MΦ in DNA repair in MM. Here, we found that MΦ regulated DNA repair in MM cells by the CXCL5/8-CXCR2 axis. By promoting non-homologous end joining rather than homology-directed repair, MΦ increased the probability of chromosomal translocations in MM cells. Furthermore, clinical data confirmed that MΦ are closely associated to the increased genetic variations of MM patients' primary cells. The study elucidates a mechanism by which MΦ regulate DNA repair in MM in the microenvironment and provides a potentially new target to counter MM progression.

Introduction

Multiple myeloma (MM) is a hematological malignancy of B cells in the terminal differentiation stage.[1] With the continuous development of treatments, the prognosis of MM patients has significantly improved.[2] Nevertheless, MM is still an incurable disease, and most patients experience progression and relapse.[3] The mechanism of the development of MM has not been fully elucidated.

Stimulated by infection, inflammation and other biochemical processes, DNA damage and genome instability may occur in plasma cells, leading to increasing incidence of specific genetic mutations and driving the gradual development of monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM) and MM.[1,4] As the disease progresses to MM, new cytogenetic abnormalities continue to occur and accumulate. DNA double-strand breaks (DSB), the most dangerous type of DNA damage, are known to induce many genetic aberrations, such as insertion and deletions and chromosomal translocations, especially when DSB repair is defective. DNA DSB are repaired mainly by homologous recombination (HR) and non-homologous end joining (NHEJ), which are the two major evolutionarily conserved repair pathways in mammalian cells.

There is now extensive evidence that the bone marrow immune microenvironment plays an important role in promoting the survival, proliferation, and drug resistance of MM cells.[5] The role of MM-associated macrophages (MΦ) in tumor immune surveillance and escape has received increasing attention. MM cells also act on MΦ by polarizing MΦ towards the M2 phenotype to promote MM cell survival and evolution.[6-8]

MΦ promote the survival and development of MM cells through various related cytokines and surface molecules. C-X-C motif chemokine ligands 5[9]/8[10] (CXCL5/8) are important chemokines in the tumor microenvironment that are expressed by many immune cells including MΦ. CXCL5/8 binds to receptors such as C-X-C motif chemokine receptor 2 (CXCR2) and plays a role in angiogenesis, tumor growth, and metastasis.[9,10] However, the CXCL5/8-CXCR2 axis is rarely reported in MM disease, and its impact on DNA repair patterns is also unknown.

Here, we found that MΦ facilitate error-prone DNA repair in MM cells and increase the genomic instability of MM cells, thereby promoting the progression of MM. These results shed a light on the role of MΦ in regulating DNA repair in MM cells and inducing genomic instability and genetic complexity of these MM cells.

Methods

The study adheres to the Declaration of Helsinki, and was approved by the Animal Experimental Ethical Inspection (ref. no. 2017-584) and Ethical Committee (ref. no. 2017-634) of the First Affiliated Hospital, Zhejiang University, School of Medicine.

Multiple myeloma xenograft model

Construction of a NOD-SCID mouse xenograft model with multiple myeloma cells

Four-week-old NOD-SCID mice were raised in the specific pathogen-free animal room of Zhejiang Academy of Medical Sciences. We collected well-grown ARP-1 cells in logarithmic growth phase, and injected ARP-1 cells subcutaneously into the left side of NOD-SCID mice (1×10^6 cells/100 μL per mouse).

X-ray irradiation experiment

Approximately 10 days after NOD-SCID mice were subcutaneously transplanted with ARP-1 cells, when the tumor reached approximately 100 mm^3, mice were randomly divided into subgroups. Mice in the irradiated groups were irradiated with a 5 Gy irradiator (PXi, USA). As for details of the mice experiments in Figure 2, immediately after irradiation, mice in the MΦ group were intratumorally injected with MΦ (1.8×10^6 cells/100 μL per mouse), and mice in the control and irradiated-only groups were injected with 100 μL per mouse of serum-free RPMI 1640 medium. The tumor tissues were collected after 24 hours. As for other details of the mice experiments in Figure 4, mice in the MΦ groups were intratumorally injected with MΦ (1.8×10^6 cells/100 μL per mouse) per 3 days, and the tumor tissues were collected 7 days after X-Ray. CXCR2 antagonist (0.25 mg/ mouse, Cat#HY-147392), CXCL5 neutralizing antibody (10 μg/mouse, Cat#254-XB) and CXCL8 neutralizing antibody (10 μg/mouse, Cat#208-IL) were adopted in corresponding subgroups.

Cas9 lentivirus (LV-Cas9) and gA2+4 lentivirus (LV- gA2+4) were used to detect NHEJ repair in the subcutaneous tumors of MM xenograft mice. After NOD-SCID mice with subcutaneous tumor formation were randomly divided into subgroups. Negative lentivirus (1.0×10^8 Tu/100 μL per mouse), LV-Cas9 and LV-gA2+4 (LV-Cas9 0.5×10^8 Tu + LV-gA2+4 0.5×10^8 Tu/100 μL per mouse) were adopted in corresponding subgroups. Twenty-four hours after lentivirus injection, mice in the MΦ group were injected with the MΦ (1.8×10^6 cells/100 μL per mouse), while the control group mice were injected with 100 μL of serum-free RPMI 1640 medium. Seventy-two hours later, subcutaneous tumors were obtained.

Statistical analysis

The measurement data are expressed as mean ± standard deviation (mean ± SD). Unpaired two-tailed *t* test, paired two-tailed *t* tests, χ^2 tests and Mann-Whitney U tests were used to compare the two groups of samples. For details, refer to the figure legends. $P<0.05$ was regarded as statistically significant, and $0.01\leq P<0.05$, $0.001\leq P<0.01$, and $P<0.001$were regarded as *, **, and ***, respectively. Otherwise, the differences were regarded as not significant (NS). Additional methods and analyses are provided in the *Online Supplementary Appendix.*

Results

Myeloma-associated macrophages facilitate double strand break repair in multiple myeloma cells *in vitro*

Single-cell RNA-sequencing (RNA-seq) of bone marrow cells from MM patients identified a higher macrophage ratio in relapsed and refractory MM (RRMM) than in newly diagnosed MM (NDMM) (*Online Supplementary Figures S1A, B*). Given the protection of MM cells by MΦ,[8,11] we further found that in co-culture, MΦ reduced γH2AX (DNA damage marker) (*Online Supplementary Figure S1C-H*) while other non-tumor cells in the bone marrow had no such effect (*Online Supplementary Figure S1I-K*). MΦ also protected MM cells from chemotherapeutic agent-induced DNA damage and apoptosis (*Online Supplementary Figure S2A-P*). We hypothesized that MΦ may modulate the choice of the DSB repair pathway in MM cells. To test this hypothesis, MM cells were treated with DSB-inducing X-ray irradiation or bleomycin (BLM) for 1 hour (h) and co-cultured with or without MΦ. Analysis of γH2AX by western blot revealed that the γH2AX level reached a peak at 1 h or 2 h and then gradually decreased in the absence of MΦ, whereas the intensity of γH2AX decreased comparatively faster in the presence of the MΦ (Figure 1A), indicating a faster DNA repair in the MΦ co-culture.

By immunofluorescence for γH2AX foci, we found that the percentage of $\gamma H2AX^+$ MM cells (i.e., cells with more than 7 γH2AX foci) in MΦ co-culture decreased faster than in the control group (*Online Supplementary Figures S1B, S3* and *S4*). ARP-1 (Figures 1B; *Online Supplementary Figure S3A, B*) and MM.1S (*Online Supplementary Figure S3C, D*) cells showed differences between these two groups starting at 4 h, and CAG cells showed clear differences starting at approximately 2 h (*Online Supplementary Figure S4A, B*). Furthermore, this difference became more significant with time. These results indicated that γH2AX in MM cells was degraded faster in the MΦ co-culture group than in the

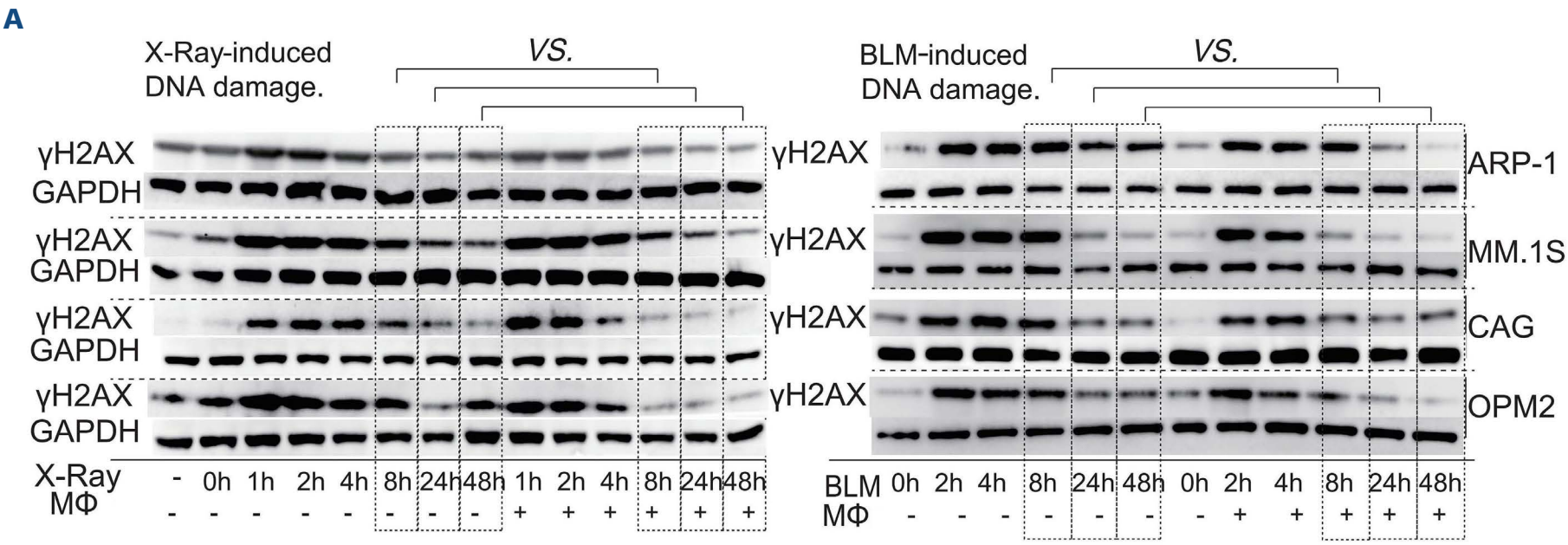
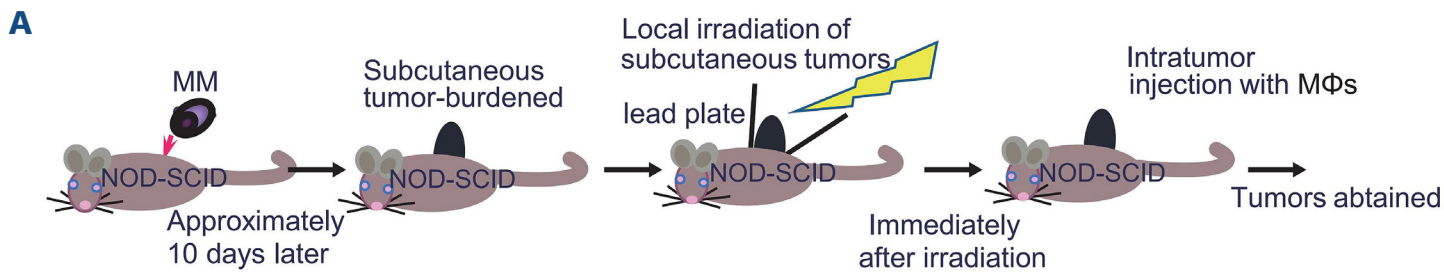
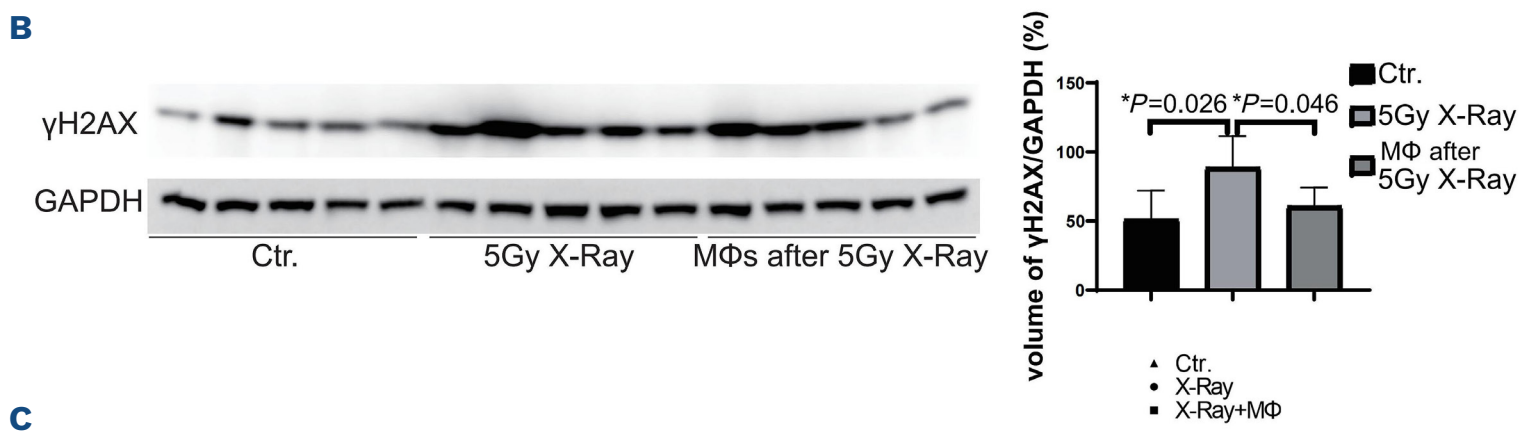


Figure 1. Multiple myeloma-associated macrophages facilitate DNA double-strand break repair in multiple myeloma cells *in vitro*. (A) Western blot analysis showed γH2AX in ARP-1 cells treated with double-strand break (DSB)-inducing X-ray irradiation for 5 Gy or bleomycin (BLM) 5 μg/mL for 1 hour (h) and co-cultured with or without multiple myeloma(MM)-associated macrophages (MΦ). The γH2AX level reached a peak at 1 h or 2 h and then gradually decreased in the absence of MΦ, whereas the intensity of γH2AX decreased comparatively faster, especially 8 h after DNA damage, in the presence of MΦ. (B) An immunofluorescence experiment was performed for γH2AX foci in ARP-1 cells with DNA-inducing X-ray irradiation at 5 Gy with or without MΦ. A high-content imaging system was used to automatically capture the nuclei and γH2AX foci and count the number of ARP-1 cells with more than 7 γH2AX foci (marked as γH2AX$^+$) (unpaired two-tailed *t* test). (C) Comet assay images showed damaged DNA in MM cells treated with X-ray irradiation for 5 Gy. CASP_1.2.3 software was applied to analyze the comet pictures and calculate the proportions of damaged DNA of *ARP-1* (unpaired two-tailed *t* test). 0.01≤*P*<0.05; 0.001≤*P*<0.01; and *P*<0.001 were regarded as *; **; and ***, respectively. Ctr: control.





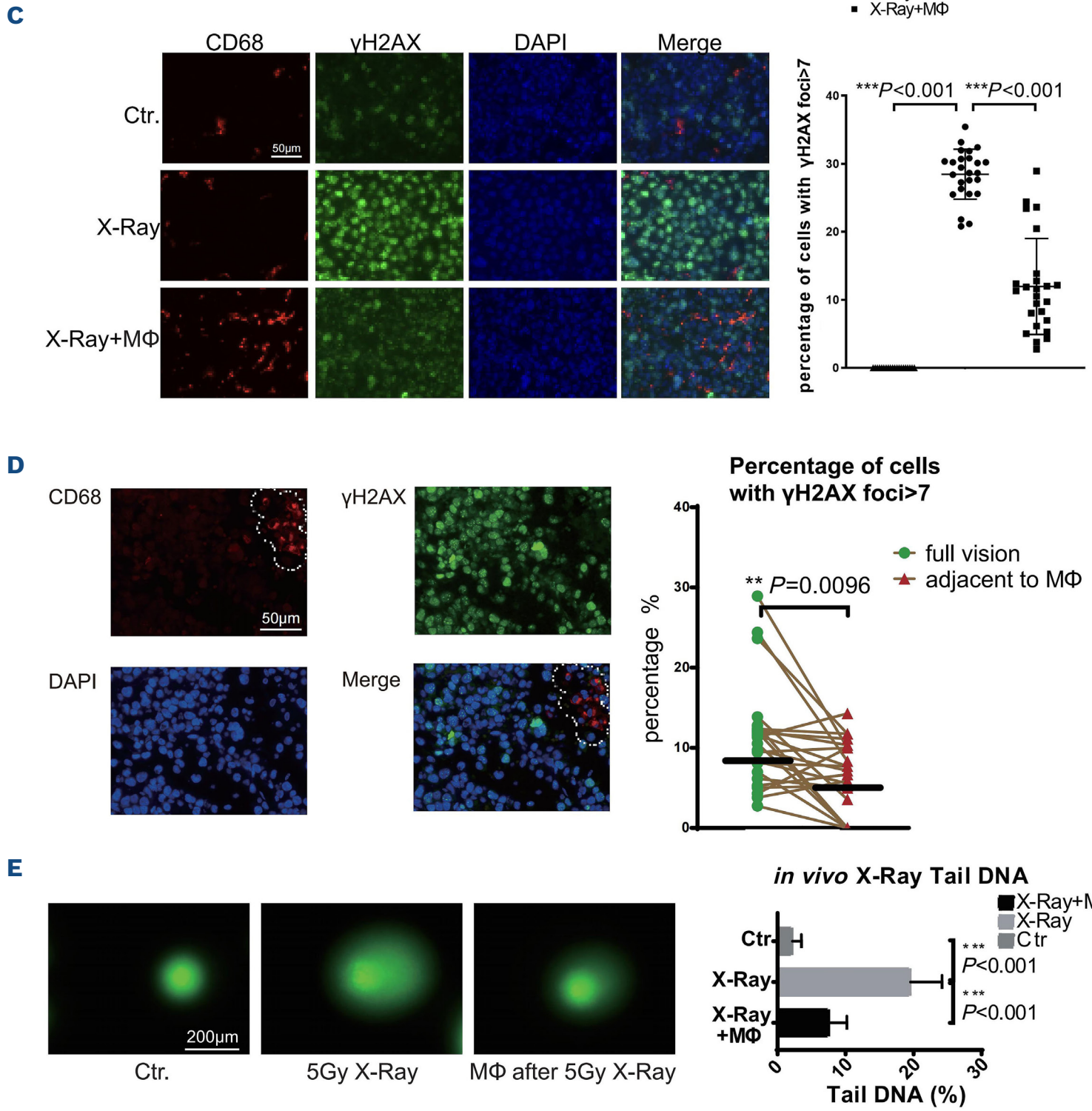


Figure 2. Multiple myeloma-associated macrophages protect irradiated multiple myeloma cell-derived xenografts *in vivo* by facilitating DNA double-strand break repair in multiple myeloma cells. (A) The workflow of the experiment *in vivo* to detect the effect of multiple myeloma (MM)-associated macrophages (MΦ) injected into irradiated tumors. (B) Western blot showing γH2AX in tumor cells from MM xenografts. ImageJ was used to test the volume of the western blot bands, which showed that the av-

Continued on following page.

erage volume of γH2AX bands was lower in the MΦ group than the control group. (C) Immunofluorescence images showed γH2AX foci from tumor cells in the control (Ctr.), X-ray irradiated-only (5 Gy X-ray) and MΦ groups (MΦ after 5 Gy X-ray). The number of tumor cells with more than 7 γH2AX foci (referred to as γH2AX$^+$) in the MΦ group was much lower than that in the irradiated-only group. Twenty-five views from 5 mice in the X-ray irradiated-only and MΦ groups, and 20 views in the control group were analyzed (unpaired two-tailed *t* test). (D) Immunofluorescence images showing the γH2AX foci of the tumor tissues in the MΦ group. MΦ were marked by CD68. The number of γH2AX$^+$ tumor cells adjacent to the MΦ was significantly lower than the average value of the entire field of view (unpaired two-tailed *t* test). (E) Comet assay images showed damaged DNA in each group with the tumors treated with X-ray irradiation for 5 Gy. CASP_1.2.3 software was applied to analyze the comet pictures and calculate the proportions of damaged DNA. Twenty-five views of comet images in each group were analyzed (unpaired two-tailed *t* test).

control group. It is possible that co-cultured MΦ promote the repair of DNA DSB in MM cells.

Using comet assays, we directly analyzed damaged DNA in irradiated MM cells. Comet tails were evident in ARP-1, MM.1S and CAG cells treated with X-ray irradiation, which were much shorter in co-culture with MΦ at 4 h after irradiation compared to culture alone (Figures 1C; *Online Supplementary Figure S5A, B*). This further indicates that co-cultured MΦ facilitate the repair of DSB in MM cells.

Myeloma-associated macrophages protect irradiated multiple myeloma cell-derived xenografts *in vivo* by facilitating double-strand repair repair in multiple myeloma cells

To determine the effect of MΦ on DSB repair in MM cells *in vivo*, we used NOD-SCID mice injected with ARP-1 cells to establish subcutaneous MM cell-derived xenografts (Figure 2A). We analyzed the induction and degradation of γH2AX in tumor cells from MM xenografts by western blotting. The γH2AX level of MΦ injection group was generally lower than that of the group without MΦ injection (Figure 2B). The number of irradiation-induced γH2AX$^+$ cells in the MΦ group was much lower than that in the group without MΦ injection (Figure 2C) by immunofluorescence. In addition, in the tumor tissue, γH2AX foci in MM cells adjacent to MΦ were much fewer than that in the other parts of the field (Figure 2D). By calculating the proportion of γH2AX$^+$ MM cells, we found that the number of γH2AX$^+$ MM cells adjacent to MΦ was significantly lower than the average number in the whole field (Figure 2D).

Comet assay was also performed and after irradiation, the comet tails of MM cells in the MΦ group were shorter than those in the control group (Figure 2E). Quantification of tail DNA indicated that the level of damaged DNA in the MΦ group was significantly lower (Figure 2E). Consistent with these *in vitro* results, these findings indicate that MΦ also promote DNA repair in MM cells *in vivo* after DNA damage, and imply that direct contact between MΦ and MM cells is important for promoting DSB repair in MM cells after DNA damage.

The CXCL5/8-CXCR2 axis mediated the effect of myeloma-associated macrophages on multiple myeloma cells in DNA repair

The single-cell RNA-seq of bone marrow cells from MM patients identified MΦ (*Online Supplementary Figure S6A-D*). MΦ differed between RRMM and NDMM patients (*Online Supplementary Figure S6E*), which indicated the changes occurring among myeloma progression. Analyzing differential expressing genes of MΦ showed that chemokines varied, with higher expression in RRMM than in NDMM (Figure 3A). To further investigate the role of the chemokines, we performed RNA-seq analysis on MΦ co-cultured with or without MM cells (ARP-1, after DNA damage or not). The heatmap showed that the expression of *CXCL* in MΦ co-cultured with ARP-1 increased (*Online Supplementary Figure S6F*). Real-time quantitative ploymerase chain reaction (RT-qPCR) results showed the upregulation of CXCL1/2/3/5/6/8/10 transcription in MΦ by co-cultured by ARP-1 cells (Figure 3B; *Online Supplementary Figure S6G*). On the other hand, RNA-seq analysis of ARP-1 cells revealed an increased expression of *CXCR2*, the common receptor for CXCL3/5/6/8, after DNA damage induced by X-ray and bleomycin (*Online Supplementary Figure S6H*). RT-qPCR validation confirmed the upregulation of *CXCR2* expression in ARP-1 cells after DNA damage (Figure 3C; *Online Supplementary Figure S6I*). To validate the role of the CXCR2 in MΦ-mediated DNA repair in MM cells, we added a CXCR2 antagonist to the co-culture system. The results showed that the CXCR2 antagonist attenuated the protective effect of MΦ on ARP-1 DNA repair after damage (Figure 3D). To search for effective ligands for CXCR2, CXCL3/5/6/8 recombination was adopted. Addition of CXCL5 or CXCL8 recombinant proteins to ARP-1 cells resulted in enhanced DNA repair (Figure 3E), whereas CXCL3 and CXCL6 recombinant proteins did not work as other ligands for CXCR2 in CXCL3/5/6/8 (*Online Supplementary Figure S6J*). Thus, the CXCL5/8-CXCR2 axis was chosen for the following studies.

To confirm the extracellular secretion of CXCL5/8 by MΦ, CXCL5/8 in the supernatants of MΦ cultures was measured by enzyme-linked immunosorbant assay. The results again verified that the levels of CXCL5/8 in the supernatants were significantly increased after co-culture with ARP-1 cells (Figure 3F). Similarly, knocking down *CXCL5/8* in MΦ by small interfering RNA (siRNA) weakened their promotive effect on ARP-1 DNA repair (Figure 3G; *Online Supplementary Figure S6K*). Furthermore, combined neutralization antibodies of CXCL5 and CXCL8 in the co-culture system partially attenuated the pro-repair effect of MΦ on ARP-1 cells (Figure 3H). Overall, these results suggest that the CXCL5/8-CXCR2 axis is a possible mechanism for MΦ to promote DNA repair in MM cells after damage.

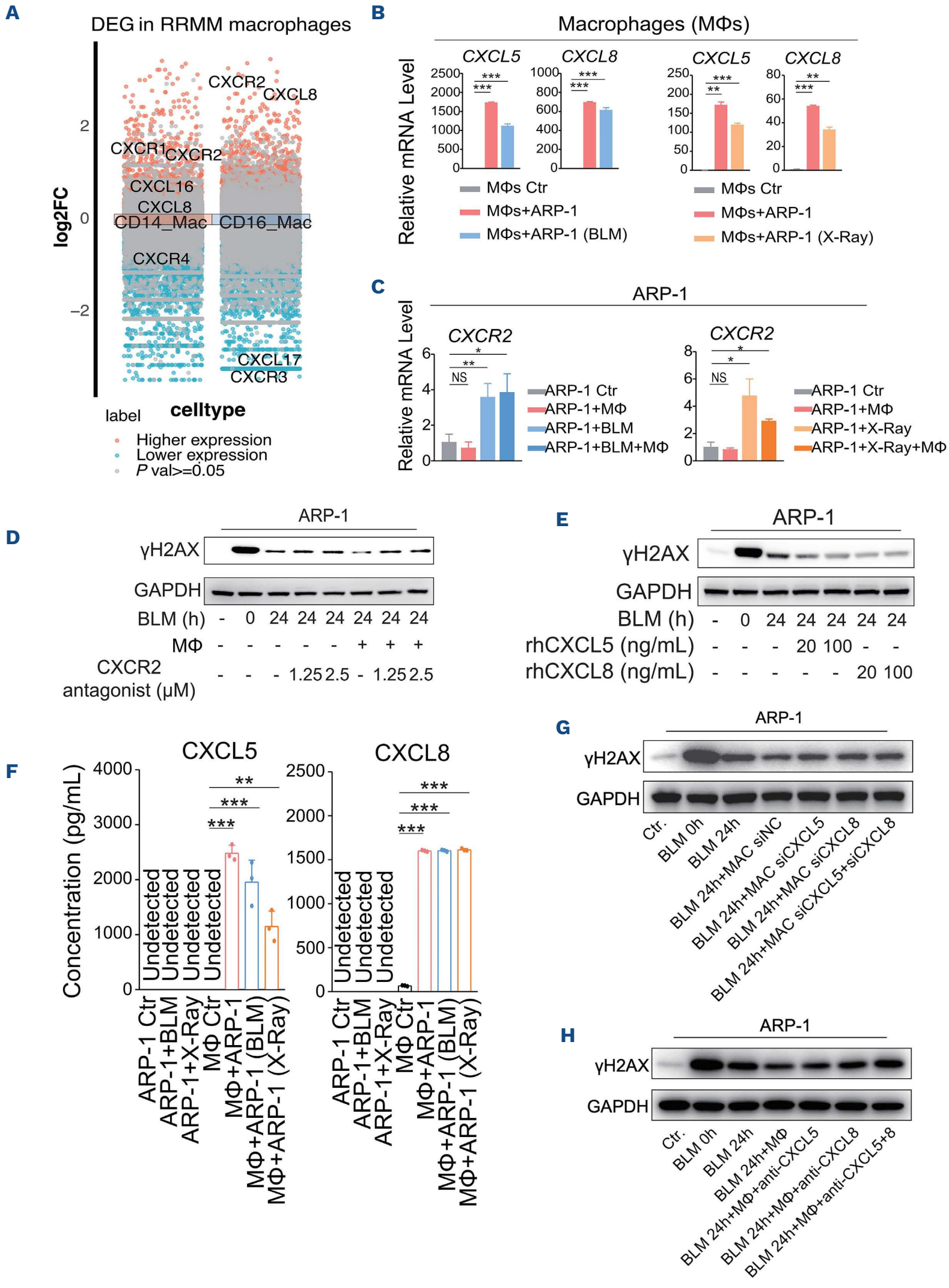


Figure 3. The CXCL5/8-CXCR2 axis mediated the effect of multiple myeloma-associated macrophages on multiple myeloma cells in DNA repair. (A) Single-cell RNA-sequencing of bone marrow cells from newly diagnosed multiple myeloma (NDMM) and relapsed and refractory MM (RRMM) were conducted. As 2 kinds of macrophages were observed, 1 expressing CD14 (CD14_MAC) and the other primarily expressing CD16 (CD16_MAC) (*Online Supplementary Figure S6A-D*), we analyzed differential expressing genes of

Continued on following page.

both macrophages in RRMM and NDMM cells. Chemokines varied a lot, such as *CXCL8*, which was expressed higher both in CD14_MAC and CD16_MAC in RRMM than in NDMM. (B) Real-time quantitative polymerase chain reaction (RT-qPCR) results showed the upregulation of *CXCL5/8* transcription in MΦ by co-cultured by ARP-1 cells (unpaired two-tailed *t* test). (C) RT-qPCR showed the upregulation of *CXCR2* expression in ARP-1 cells after DNA damage (unpaired two-tailed *t* test). (D) The CXCR2 antagonist attenuated the promotive effect of MΦ on ARP-1 DNA repair after damage. (E) Addition of CXCL5 or CXCL8 recombinant proteins to ARP-1 cells resulted in enhanced DNA repair. (F) CXCL5/8 in the supernatants of MΦ co-cultured with ARP-1 increased by enzyme-linked immunosorbant assay (unpaired two-tailed *t* test). (G) Knocking-down *CXCL5/8* in MΦ by small interfering RNA (siRNA) weakened their promotive effect on ARP-1 DNA repair. (H) The addition of neutralizing antibodies against CXCL5 (CXCL5 NAb) and CXCL8 (CXCL8 NAb) to the co-culture system inhibit the promotive effect of MΦ on ARP-1 DNA repair. $0.01 \le P < 0.05$, $0.001 \le P < 0.01$, and $P < 0.001$ were regarded as *, **, and ***, respectively.

Interference with the CXCL5/8-CXCR2 axis inhibited the protective effect of macrophages on multiple myeloma cells *in vivo*

To further investigate the role of CXCL5/8-CXCR2 axis in the interaction between MΦ and MM cells, we conducted experiments using NOD-SCID mice (Figure 4A). It was shown that tumor were smaller in X-Ray group than that in the control group, and that irradiated tumors with MΦ injection were larger than those without MΦ injection, while CXCR2 antagonist and CXCL5/8 neutralizing antibody reduced the protective effect of MΦ on MM (Figure 4B). The volume of tumors were evaluated and the above result remained significant (Figure 4C).

Immunohistochemical staining marked MΦ, CXCL5/8, CXCR2 and γH2AX, which showed the γH2AX in the X-Ray group was significantly higher than that in the control group, and MΦ injection reduced the γH2AX of MM cells (*Online Supplementary Figure S7A*). CXCR2 antagonist and CXCL5/8 neutralizing antibody weaken the promoting effect of MΦ on DNA repair in MM cells *in vivo* (*Online Supplementary Figure S7A*). This suggested that MΦ promoted MM cell proliferation and survival after DNA damage *in vivo*, and CXCL5/8-CXCR2 axis played a role in the effect.

Myeloma-associated macrophages promote mutagenic non-homologous end joining in multiple myeloma cells *in vitro*

DNA DSB and their repair, including HR and NHEJ pathways,

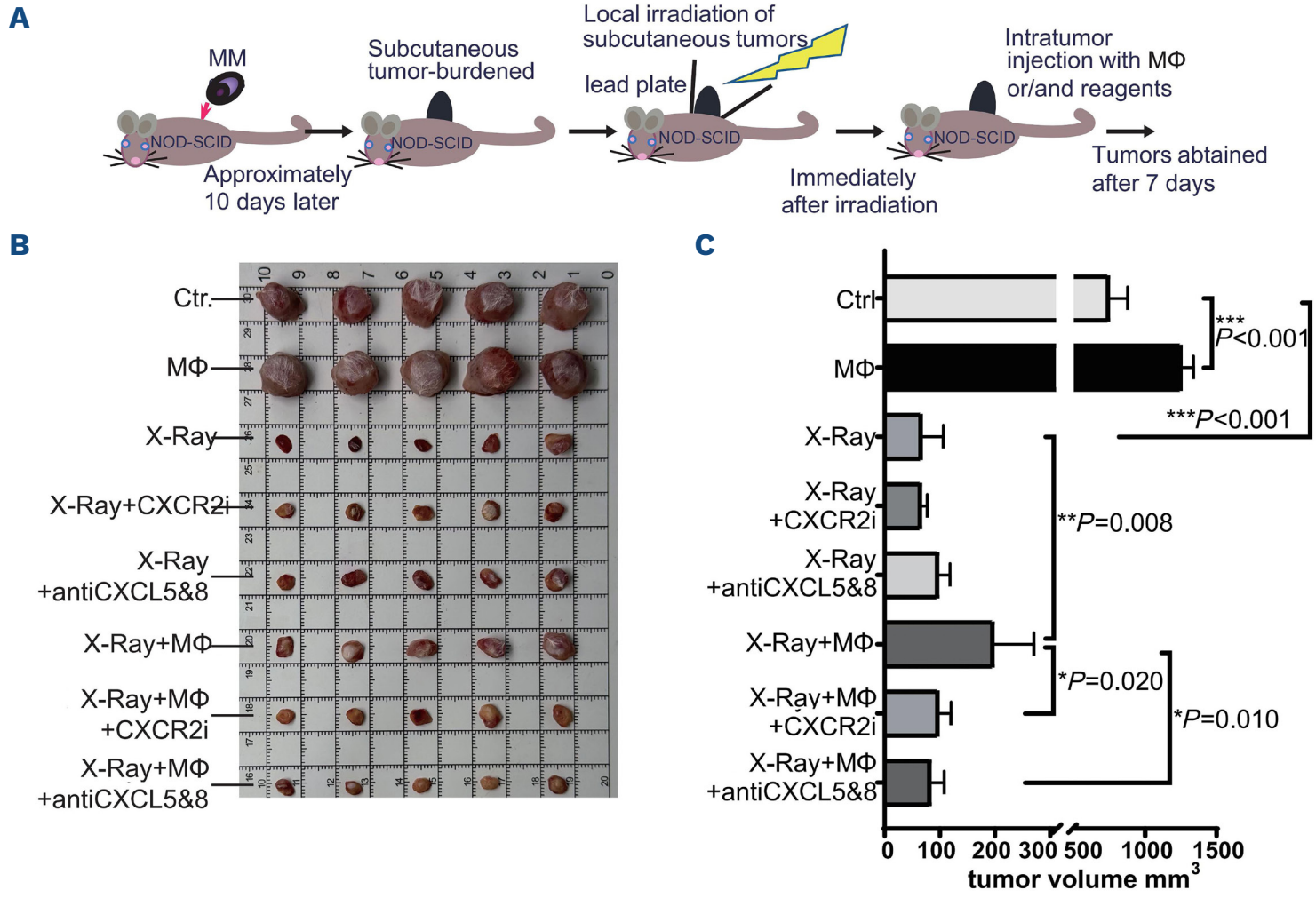


Figure 4. Interference with the CXCL5/8-CXCR2 axis inhibited the protective effect of multiple myeloma-associated macrophages on multiple myeloma cells *in vivo*. (A) The workflow of the experiment *in vivo*. (B) Image of subcutaneous tumors from NOD-SCID mice in different groups. (C) The volume=1/2 (length × width^2). Tumor were smaller in X-Ray group than that in the control group. Irradiated tumors with multiple myeloma (MM)-associated macrophages (MΦ) injection were larger than those without MΦ injection, while CXCR2 antagonist and CXCL5/8 neutralizing antibody declined the protective effect of macrophages on MM. Unpaired two-tailed *t* test, $0.01 \le P < 0.05$, $0.001 \le P < 0.01$, and $P < 0.001$ were regarded as *, **, and ***, respectively.

play an important role in the clonal evolution of MM cells.[19] To explore which mechanisms were involved in MΦ promotion of DSB repair in co-cultured MM cells, we first used the osteosarcoma cell line U2OS that harbors a single-copy HR or NHEJ reporter system in the genome as tool cells.[12,13] We found that co culturing with MΦ enhanced NHEJ rather than HR in U2OS cells (*Online Supplementary Figure S8*).

To directly detect whether MΦ regulate NHEJ in MM cells, we used the paired SpCas9-gRNA to induce site-specific DSB in MM cells[14] (Figure 5A). We analyzed the DNA cutting efficiency of paired SpCas9-sgRNA targeting AAVS1 and HBB (*Online Supplementary Figure S9A*). For detailed methods, see Guo *et al.*[14] Based on the criteria for optimal paired SpCas9-sgRNA, we selected paired AAVS1 sgRNA gA2/gA4 and paired HBB sgRNA gH1/gH5 for NHEJ assays (*Online Supplementary Figure S9B, C*).

Lentiviruses, containing expression cassettes for paired SpCas9-sgRNA targeting AAVS1 and HBB, were used to infect MM cells and genomic DNA were isolated at 3 days post infection. NHEJ repair was increased in MM cells co-cultured with MΦ (Figures 5B; *Online Supplementary Figure S9D*). Nevertheless, the frequency of accurate NHEJ in group I was reduced in the MΦ group (Figure 5B; *Online Supplementary Figure S9D*). This suggests that MΦ promote mutagenic NHEJ in MM cells. The median length of group I deletions with MΦ was 10 bp at the AAVS1 site and 11 bp at the HBB site, longer than 9 bp at either site in the control group (Figure 5C; *Online Supplementary Figure S9E*; Mann-Whitney test; $P<0.001$, respectively). Moreover, MM cells co-cultured with MΦ caused more frequent mutagenic NHEJ events with deletions of over 3 bp at the AAVS1 site (Figure 5D, 51.68% *vs*. 48.55% with or without MΦ; χ^2 test; $P<0.001$) and the HBB site *Online Supplementary Figure S9F*; 65.08% *vs*. 58.01% with or without MΦ; χ^2 test; $P<0.001$). These data indicate that MΦ promote NHEJ in MM cells and reduce accuracy.

Myeloma-associated macrophages promote mutagenic non-homologous end joining in multiple myeloma cells *in vivo*

To measure the NHEJ repair of MM cells *in vivo*, we generated subcutaneous ARP-1 cell-derived xenografts in NOD-SCID mice (Figure 6A). The subcutaneous tumor tissues were collected, as shown in *Online Supplementary Figure S10A*. Tumors in the MΦ group were larger than those without MΦ injection (*Online Supplementary Figure S10B*). This stress response to DNA cleavage and subsequent DNA repair, likely contributed to the observed tumor size reduction within "Cut" groups compared to controls. The tagged GFP expression in cell suspensions showed cells in three groups were all infected effectively (*Online Supplementary Figure S10C*).

Similar NHEJ analysis of ARP-1 cells selected by CD138 magnetic microbeads from the cell suspension of mouse tumor tissues revealed that MM cells from the MΦ group had a reduced proportion of Group I (*Online Supplementary Figure S10D*), a significantly increased frequency of NHEJ (Figure 6B), and a reduced proportion of accurate NHEJ repair (Figure 6C), consistent with our *in vitro* findings. Furthermore, the number of bases lost in the MΦ group was larger (Figure 6D, the median length of group I deletions was 25 bp *vs*. 17 bp, Mann-Whitney test; $P<0.001$). Consistently, the probability of base loss >3 bp was higher in the group with MΦ injection than in the control group (Figure 6E, 70.04% *vs*. 57.80% with or without MΦ; χ^2 test; $P<0.001$). Immunofluorescence labeling showed that MΦ reduced DNA damage caused by paired Cas9-gRNA' splicing, which meant compared with the cut group alone, the MΦ injection group showed a decrease in γH2AX (*Online Supplementary Figure S10E*). Taken together, these results indicate that MΦ also promote MM cell NHEJ repair but reduce accuracy *in vivo*.

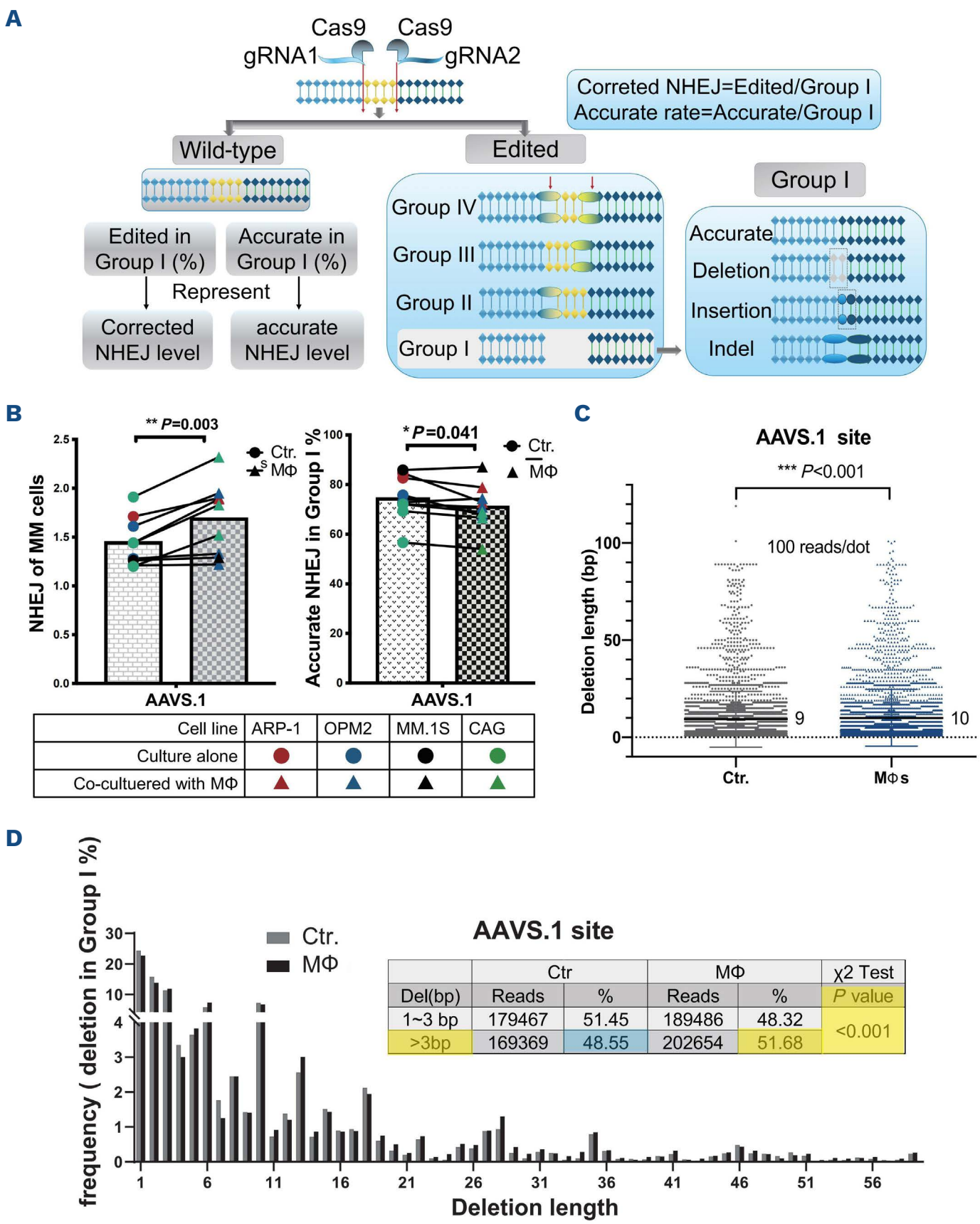
Myeloma-associated macrophages promote chromosomal translocations in multiple myeloma cells

Chromosomal translocations frequently occur in MM. We wondered whether co-culturing with MΦ alters the frequency of chromosomal translocations in MM cells. We used a pair of SpCas9-sgRNA targeting the site of *CCND1* on chromosome 11 and the site of IgH on chromosome 14 to induce reciprocal IgH-CCND1 translocations in ARP-1 cells (Figure 7A).[15] Reciprocal IgH-CCND1 translocations at the der(11) and der(14) chromosomes were detected by PCR and gel electrophoresis (Figure 7B). Using PCR bands of the β-actin gene as an internal PCR control, we found that induction of IgH-CCND1 der(14) translocations in MM cells was stronger with co-cultured MΦ than without MΦ (Figures 7C, D). Moreover, tested by next-generation sequencing sequencing, it was found that co-culture with MΦ increased this relative chromosomal translocation probability, further validating that MΦ promote chromosomal translocation in MM cells (Figure 7E).

Myeloma-associated macrophages in multiple myeloma patients are associated with cytogenetic complexity of multiple myeloma

For clinical data, we explored the relationship between MΦ and the cytogenetic complexity of MM patients. Fluorescence *in situ* hybridization (FISH) data were collected to detect the genetic abnormalities of MM cells in patients. The hospital applied IgH, 1q21, D13S319, TP53 and RB1 probes to target 14q32, 1q21, 13q14.3, 17p13.1, and 13q14.2 sites. We collected 33 bone marrow biopsy specimens and labeled the bone marrow MΦ with CD68 antibody.[16] Three examples of patients at each level are shows in Figure 8A, and Figure 8B showed the FISH results of these three patients. The cutoff level of FISH was 10% for fusion and 20% for numerical abnormalities.[17] The results showed that the higher the content of MΦ was, the higher the FISH positive rate was. In particular, the positive rates of IgH translocation and D13S319 locus deletion were much

higher in patients with MΦ contents of ++ and +++ than contents of + (Figure 8C). Among the above five FISH results, patients with ≥3 positive indicators were considered to have complex genetic abnormalities. The results showed



	Ctr		MΦ		χ2 Test
Del(bp)	Reads	%	Reads	%	*P* value
1~3 bp	179467	51.45	189486	48.32	<0.001
>3bp	169369	48.55	202654	51.68	

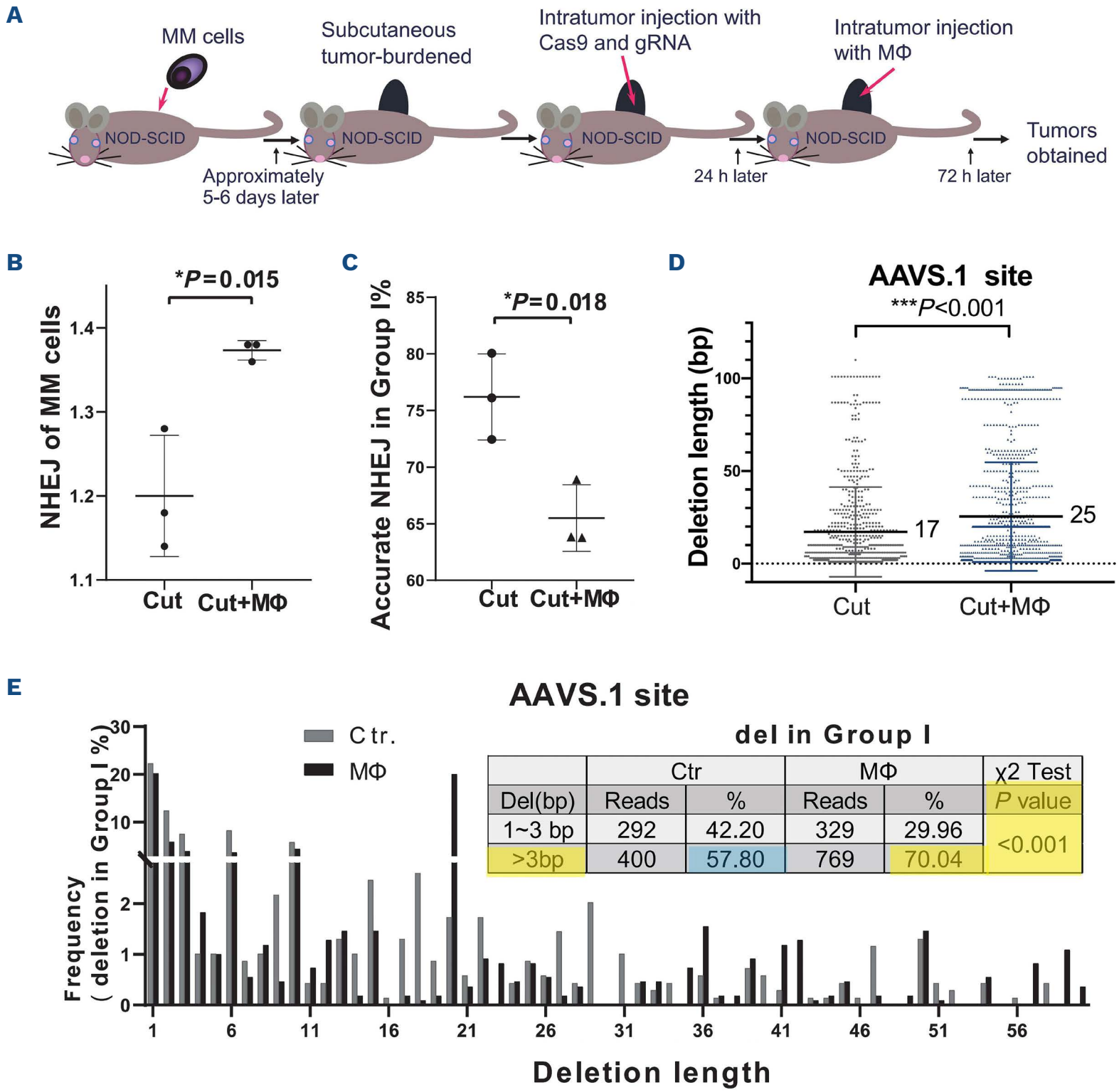
Figure 5. Multiple myeloma-associated macrophages promote mutagenic non-homologous end joining in multiple myeloma cells *in vitro*. (A) The workflow of the paired SpCas9-gRNA approach to induce site-specific double-strand breaks (DSB) in multiple myeloma (MM) cells and study non-homologous end joining (NHEJ) repair. NHEJ of DSB induced by paired SpCas9-single guide RNA (sgRNA) generates 4 groups of NHEJ products: groups I, II, III and IV.[14] Because accurate NHEJ and mutagenic NHEJ can only be distinguished and quantified in group I products, optimal paired SpCas9-sgRNA for NHEJ assays should be determined not only by efficient cutting of the paired SpCas9-sgRNA but also by a high frequency of group I. We analyzed the DNA cutting efficiency of paired SpCas9-sgRNA using targeted polymerase chain reaction (PCR) and gel electrophoresis and NHEJ repair products using next-generation sequencing of targeted PCR amplicons after transfection of HEK293T cells with paired SpCas9-sgRNA targeting AAVS1 and HBB. (B) Co-culture with MΦ increased the efficiency of overall NHEJ (paired two-tailed *t* test) at the AAVS1 site. Co-culture with MM-associated macrophages (MΦ) reduced the frequency of accurate NHEJ in group I (paired two-tailed *t* test) at the AAVS1 site. (C) The median length of group I deletions with MΦ was 10 bp at the AAVS1 site, longer than 9 bp at either site in MM cells without the MΦ co-culture (Mann-Whitney test; $P<0.001$). (D) Co-culture with MΦ caused more frequent mutagenic NHEJ events with deletions of over 3 bp at the AAVS1 site (51.68% *vs*. 48.55% with or without MΦ; χ^2 test; $P<0.001$).

the positive rates of complex genetic abnormalities (+, ++ and +++: 0.0%, 22.2% and 62.5%). Among them, the positive rate of complex genetic abnormalities with MΦ content ++ and +++ levels was higher than that of the + level group (Figure 8D). These data indicate that the content of MΦ is positively correlated with MM cytogenetic abnormalities.

In addition, MΦ were induced from peripheral blood of different patients, including healthy donors, standard/high cytogenetic risk MM patients. The results showed that these MΦ could weaken the γH2AX caused by DNA damage in MM cells, that is, they all had the effect of promoting DNA repair in MM cells. The protective effect of MΦ seemed to be better in high-risk MM patients (Figure 8E).

Discussion

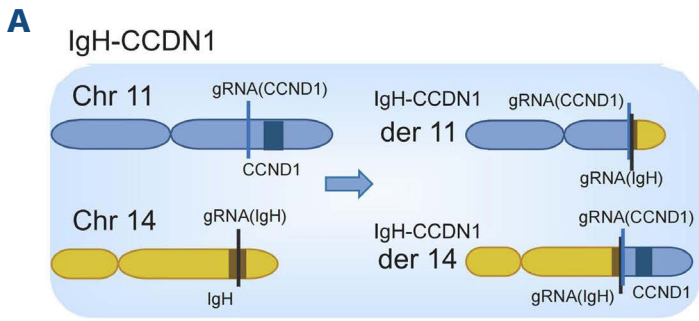
Many previous studies have shown that MΦ in MM tissues are associated with the occurrence, disease progression, and drug resistance of MM.[6,18] As a highly heterogeneous disease, MM is closely related to abnormal DNA repair

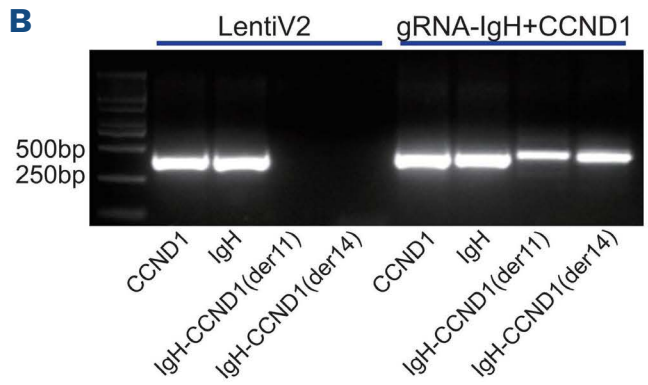


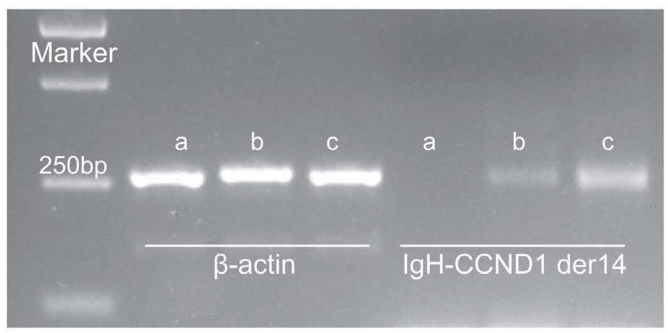
	Ctr		MΦ		χ2 Test
Del(bp)	Reads	%	Reads	%	*P* value
1~3 bp	292	42.20	329	29.96	<0.001
>3bp	400	57.80	769	70.04	

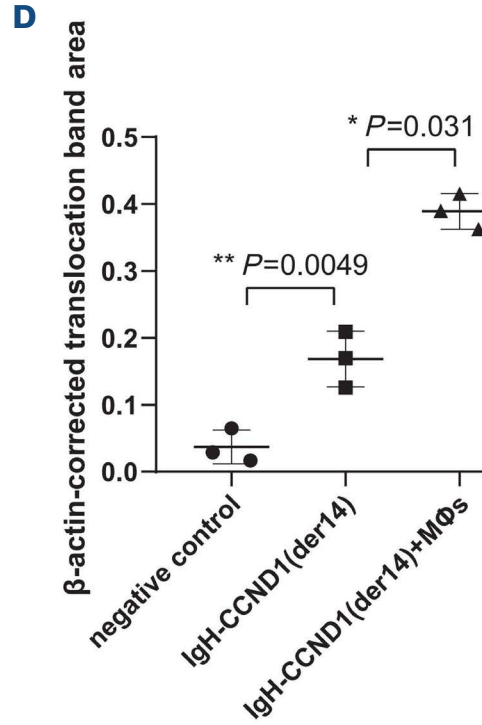
Figure 6. Multiple myeloma-associated macrophages promote mutagenic non-homologous end joining in multiple myeloma cells *in vivo*. (A) The workflow of the paired SpCas9-gRNA approach to induce site-specific double-strand breaks (DSB) *in vivo*. (B, C) The cells in the multiple myeloma (MM)-associated macrophages (MΦ) group had a significantly increased efficiency of non-homologous end joining (NHEJ) (B), and a reduced proportion of precise NHEJ repair (C) (unpaired two-tailed *t* test). (D) The median length of group I deletions in the MΦ-injected groups was 25 bp, longer than that in the gene-edited-only group (17 bp) (Mann-Whitney test; $P<0.001$). (E) The probability of base loss >3 bp in the MΦ group was higher than that in the gene-edited-only group (70.04% *vs.* 57.80% with or without MΦ; χ^2 test; $P<0.001$).

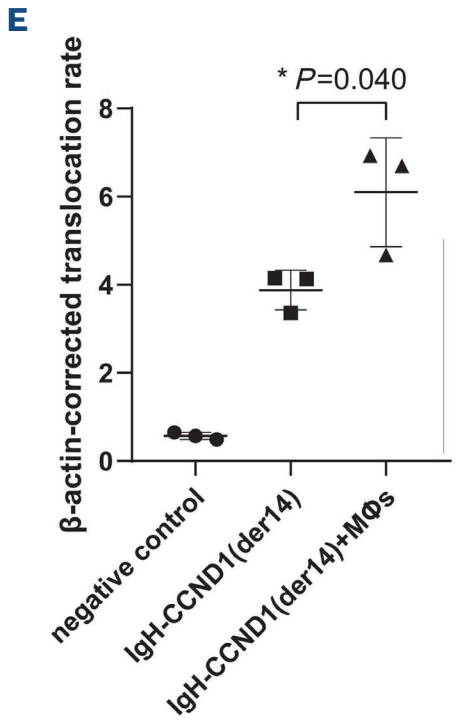
and genome instability. In other diseases, such as undifferentiated uterine sarcoma,[19] Gaucher disease,[20] gastric cancer,[21] kidney tumors[22] and other diseases, studies have indicated that MΦ can affect the clonal evolution of diseases or remodeling of tumor cells.[23] However, few studies have examined the effect of MΦ on DNA repair











Reads

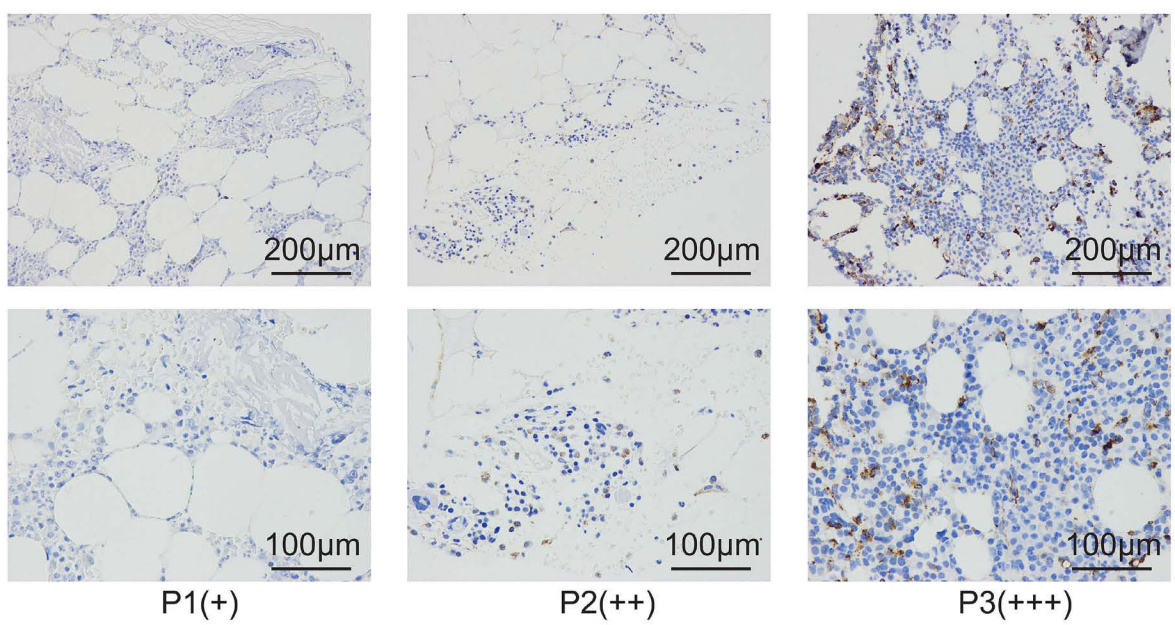
Experiment 1	I-C	749828	223064	3.36149
	I-C+MΦ	1573177	336388	4.67667
Experiment 2	I-C	1317008	317504	4.148
	I-C+MΦ	1684256	243135	6.92725
Experiment 3	I-C	1235094	299021	4.13046
	I-C+MΦ	1340784	200175	6.69806

IgH-CCND1(der14) β-actin IgH-CCND1(der14) /β-actin

Figure 7. Multiple myeloma-associated macrophages promote chromosomal translocations in multiple myeloma cells. (A) The workflow to induce IgH-CCND1 translocations. (B) Polymerase chain reaction (PCR) and gel electrophoresis were used to detect IgH-CCND1 translocations. (C) PCR and gel electrophoresis showed that the induction of IgH-CCND1 der(14) in multiple myeloma (MM) cells was stronger when co-cultured with MM-associated macrophages (MΦ). (D) ImageJ was used to test the band area, which showed that the IgH-CCND1 der(14) translocation band was larger than that in the MΦ group. (E) The IgH-CCND1 der(14) PCR products were mixed with the corresponding β-actin at a ratio of 8:1 for next-generation sequencing. The ratio of IgH-CCND1 der(14) translocation reads to β-actin sequence reads in the sample was calculated to represent the relative chromosomal translocation probability of IgH-CCND1 der(14). The results suggested that the MΦ promoted chromosomal translocation in MM cells (unpaired two-tailed *t* test).

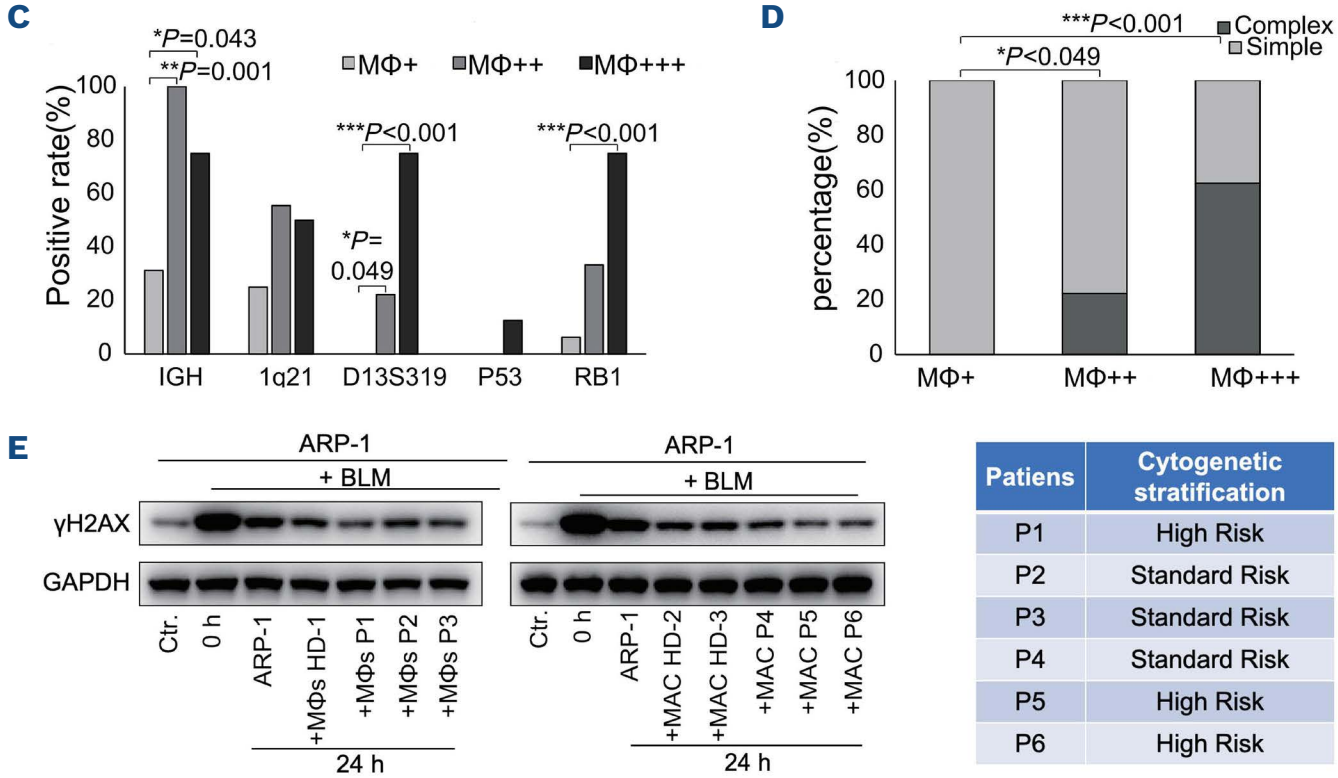
or genomic instability in MM cells. Here, we demonstrate that co-cultured MΦ protected MM cells by promoting DSB repair. CXCL5/8-CXCR2 axis may be one of the mechanisms by which MΦ exert their effect on MM cells. MΦ enhanced the NHEJ pathway, but reduced repair accuracy and promoted chromosomal translocations in MM cells. These results provide a possible mechanism by which MΦ in MM tumors facilitate the occurrence and accumulation of

A



B

FISH	IgH	1q21	D13S319	P53	RB1
P1	0	0	0	0	0
P2	0.2	0.2	0	0	0
P3	0.3	0	0.3	0	0.3



Patiens	Cytogenetic stratification
P1	High Risk
P2	Standard Risk
P3	Standard Risk
P4	Standard Risk
P5	High Risk
P6	High Risk

Figure 8. Cytogenetic complexity of multiple myeloma is closely associated with macrophages in the microenvironment. (A) Among 33 specimens, 3 examples of patients with multiple myeloma (MM)-associated macrophages (MΦ) contents in bone marrow at the +/++/+++ level. According to the positive IOD value of the immunohistochemistry films and the pathologist's judgment, the MΦ contents were divided into 3 levels: +, ++ and +++. (B) The Fluorescence *in situ* hybridization (FISH) results of these 3 patients. (C) Correlation between MΦ contents in bone marrow and the positive rate of the FISH results. The positive rate of IgH translocation was much higher in patients with MΦ contents of ++ and +++ than in patients with MΦ contents of + (P=0.001, χ^2 test; P=0.043, χ^2 test), and D13S319 locus deletion had the highest positive rate in patients with MΦ contents of ++ and +++ (P=0.049, P<0.001, χ^2 test). (D) Among the above 5 FISH results, patients with ≥3 positive indicators are considered to have complex genetic abnormalities. The positive rate of complex genetic abnormalities with MΦ content +++ level was higher than that of + level group (Π<0.001, χ^2 test). (E) MΦ were induced from peripheral blood of different patients, including healthy donors, high/standard cytogenetic risk MM patients. The results showed that these macrophages could weaken the γH2AX caused by DNA damage in MM cells, that is, they all had the effect of promoting DNA repair in MM cells. The protective effect of MΦs seems to be better in high-risk MM patients.

cytogenetic events in tumor cells and increase the genetic complexity of MM cells (*Online Supplementary Figure S11*). The overexpression of CXCL5/8-CXCR2 axis is closely related to the survival time, recurrence, and metastasis of tumor patients.[9,10] Our research has identified a role of the CXCL5/8-CXCR2 axis in information transmission during the interaction between MΦ and MM cells to promote DNA repair both *in vitro* and *in vivo*. Our conclusions not only reveal the mechanism of interaction between these two types of cells, but also uncover a new role of the CXCL5/8-CXCR2 axis in promoting tumor growth. Pharmacological targeting of this axis, whether through macrophage depletion strategies, CXCL5/8 neutralization, or CXCR2 inhibition, could disrupt this pro-malignant crosstalk. Notably, several CXCR2 antagonists have already entered clinical trials for solid tumors,[24-26] suggesting translational feasibility in MM.

HR and NHEJ are the main methods to repair DSB. Once HR and NHEJ are disrupted, various genetic events may occur and even lead to cell death.[27] To further study the effect of MΦ on NHEJ repair in MM cells, we used the paired gRNA-CRISPR/Cas9 system developed by Guo *et al.* in 2018 to detect NHEJ of MM cell endogenous genes.[14] Innovatively, we constructed and used an *in vivo* mouse model to detect the genome of NHEJ. We were pleasantly surprised to find that MΦ indeed caused changes in NHEJ repair in MM cells, not only increasing the overall NHEJ repair level but also reducing precise repair and increasing the length of base loss.

Studies have shown that in human cells, the last step of chromosomal translocation is mediated by classical NHEJ (c-NHEJ).[28] MM is a highly heterogeneous hematological malignancy, and chromosomal translocations represented by IgH translocations play an important role in the occurrence and development of MM.[29] Here, we further explored the influence of MΦ on the chromosomal translocation of MM cells. In our study, IgH-CCND, which is common in MM, was used as a tool site to detect chromosomal translocation. We clearly showed that MΦ promote chromosomal translocation of MM cells.

During continuous proliferation of tumor cells, the probability of endogenous DNA damage due to replication pressure increases.[30,31] The DNA repair process will increase the probability of genetic events.[32,33] The existence of MΦ was also conducive to the survival of MM cells, enhancing survival when chemotherapy-drugs caused genome instability in MM cells. These findings are consistent with Zheng and Chen's research conclusions that MΦ protect MM cells.[8,18] Therefore, from another perspective, MΦ maintain more MM cells that have completed DNA repair in the cell population. These MM cells undergoing DNA damage and repair have survived, and the probability that MM cells carrying mutant genes and translocated chromosomes is greater than that of the wild-type population. In other words, MΦ might promote the retention of MM cells with unstable genomes in MM cell population. We directly reveal the impact of microenvironmental cells on the DNA repair of MM cells, which is exciting.

We recognize that one of the shortcomings of the research is that some of the differences in results are somewhat modest. However, in patients, the microenvironment is the niche for MM cells to survive, and the continuous influence of microenvironmental cells on MM cells takes effect.[34,35] MΦ continue to affect MM cells in the bone marrow microenvironment, and over time, the effects accumulate, even if MΦ promote the instability of the MM cell genome to a small extent, and provide a mutant gene pool for MM cells to escape from therapeutic drugs. The high heterogeneity of MM is beneficial for the survival of tumor cell populations under the natural selection.[36,37]

In summary, our research demonstrated that MΦ promote abnormal NHEJ repair and the chromosomal translocation of MM cells. Clinical data showed that the content of MΦ was positively correlated with the cytogenetic abnormalities of MM patients. The results indicate that MΦ promote genomic complexity of MM cells. The CXCL5/8-CXCR2 axis was speculated to transduce signaling during the interaction between MΦ and MM cells. Therapeutic interventions, including depletion of MΦ, blockade of CXCL5/8, or CXCR2 antagonism, may effectively abrogate oncogenic signaling network of MM, serving as new treatment strategies. Our study elucidates one of the mechanisms affecting DNA repair in MM cells from the perspective of MΦ in the microenvironment, and provides a new insight to delay the MM progression.

Disclosures

No conflicts of interest to disclose.

Contributions

ZC and AX conceived the project and supervised the study and manuscript writing. MD, DH and JZ performed experiments, analyzed data, and wrote the manuscript. HY and HC assisted in data analysis. EZ, YF, and JH assisted in experiments and manuscript writing. XH, GC, XS, and HG provided assistance in experiments. FC evaluated the immunohistochemical slides. HW provided assistance in data analysis.

Acknowledgments

The authors are grateful to the other members of Cai and Xie's laboratories for helpful discussions.

Funding

This work was supported by National Natural Science Foundation of China (no. 82100212 to MD, no. 82173267 to ZC, no. 32371348 to AX, and no. 32071439 to YF) and Zhejiang Provincial Natural Science Foundation Key Project (Jingsong He LZ22H160009).

Data-sharing statement

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

References

1. Kyle RA, Rajkumar SV. Multiple myeloma. N Engl J Med. 2004;351(18):1860-1873.
2. Rajkumar SV, Kumar S. Multiple myeloma: diagnosis and treatment. Mayo Clinic Proceedings. 2016;91(1):101-119.
3. Laubach J, Garderet L, Mahindra A, et al. Management of relapsed multiple myeloma: recommendations of the International Myeloma Working Group. Leukemia. 2016;30(5):1005-1017.
4. Anuja K, Roy S, Ghosh C, Gupta P, Bhattacharjee S, Banerjee B. Prolonged inflammatory microenvironment is crucial for pro-neoplastic growth and genome instability: a detailed review. Inflamm Res. 2017;66(2):119-128.
5. Podar K, Chauhan D, Anderson KC. Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia. 2009;23(1):10-24.
6. Gutierrez-Gonzalez A, Martinez-Moreno M, Samaniego R, et al. Evaluation of the potential therapeutic benefits of macrophage reprogramming in multiple myeloma. Blood. 2016;128(18):2241-2252.
7. De Beule N, De Veirman K, Maes K, et al. Tumour-associated macrophage-mediated survival of myeloma cells through STAT3 activation. J Pathol. 2017;241(4):534-546.
8. Chen J, He D, Chen Q, et al. BAFF is involved in macrophage-induced bortezomib resistance in myeloma. Cell Death Dis. 2017;8(11):e3161.
9. Zhang W, Wang H, Sun M, et al. CXCL5/CXCR2 axis in tumor microenvironment as potential diagnostic biomarker and therapeutic target. Cancer Commun (Lond). 2020;40(2-3):69-80.
10. Cambier S, Gouwy M, Proost P. The chemokines CXCL8 and CXCL12: molecular and functional properties, role in disease and efforts towards pharmacological intervention. Cell Mol Immunol. 2023;20(3):217-251.
11. Yan H, Dong M, Liu X, et al. Multiple myeloma cell-derived IL-32gamma increases the immunosuppressive function of macrophages by promoting indoleamine 2,3-dioxygenase (IDO) expression. Cancer Letter. 2019;446:38-48.
12. Dellino GI, Palluzzi F, Chiariello AM, et al. Release of paused RNA polymerase II at specific loci favors DNA double-strand-break formation and promotes cancer translocations. Nat Genet. 2019;51(6):1011-1023.
13. Panchakshari RA, Zhang X, Kumar V, et al. DNA double-strand break response factors influence end-joining features of IgH class switch and general translocation junctions. Proc Natl Acad Sci U S A. 2018;115(4):762-767.
14. Guo T, Feng YL, Xiao JJ, et al. Harnessing accurate non-homologous end joining for efficient precise deletion in CRISPR/Cas9-mediated genome editing. Genome Biol. 2018;19(1):170.
15. Tsuyama N, Abe Y, Yanagi A, et al. Induction of t(11;14) IgH enhancer/promoter-cyclin D1 gene translocation using CRISPR/Cas9. Oncol Lett. 2019;18(1):275-282.
16. Takeya M, Komohara Y. Role of tumor-associated macrophages in human malignancies: friend or foe? Pathol Int. 2016;66(9):491-505.
17. Ross FM, Avet-Loiseau H, Ameye G, et al. Report from the European Myeloma Network on interphase FISH in multiple myeloma and related disorders. Haematologica. 2012;97(8):1272-1277.
18. Zheng Y, Cai Z, Wang S, et al. Macrophages are an abundant component of myeloma microenvironment and protect myeloma cells from chemotherapy drug-induced apoptosis. Blood. 2009;114(17):3625-3628.
19. Przybyl J, Kowalewska M, Quattrone A, et al. Macrophage infiltration and genetic landscape of undifferentiated uterine sarcomas. JCI insight. 2017;2(11):e94033.
20. Aflaki E, Stubblefield BK, Maniwang E, et al. Macrophage models of Gaucher disease for evaluating disease pathogenesis and candidate drugs. Sci Transl Med. 2014;6(240):240ra273.
21. Sunakawa Y, Stremitzer S, Cao S, et al. Association of variants in genes encoding for macrophage-related functions with clinical outcome in patients with locoregional gastric cancer. Ann Oncol. 2015;26(2):332-339.
22. Pasupuleti V, Du W, Gupta Y, et al. Dysregulated D-dopachrome tautomerase, a hypoxia-inducible factor-dependent gene, cooperates with macrophage migration inhibitory factor in renal tumorigenesis. J Biol Chem. 2014;289(6):3713-3723.
23. Dobyns AE, Goyal R, Carpenter LG, Freeman TC, Longo LD, Yellon SM. Macrophage gene expression associated with remodeling of the prepartum rat cervix: microarray and pathway analyses. PloS One. 2015;10(3):e0119782.
24. Armstrong AJ, Geva R, Chung HC, et al. CXCR2 antagonist navarixin in combination with pembrolizumab in select advanced solid tumors: a phase 2 randomized trial. Invest New Drugs. 2024;42(1):145-159.
25. Schott AF, Goldstein LJ, Cristofanilli M, et al. Phase Ib pilot study to evaluate reparixin in combination with weekly paclitaxel in patients with HER-2-negative metastatic breast cancer. Clin Cancer Res. 2017;23(18):5358-5365.
26. Madan A, Chen S, Yates P, et al. Efficacy and safety of danirixin (GSK1325756) co-administered with standard-of-care antiviral (Oseltamivir): a phase 2b, global, randomized study of adults hospitalized with influenza. Open Forum Infect Dis. 2019;6(4):ofz163.
27. Weeden CE, Asselin-Labat ML. Mechanisms of DNA damage repair in adult stem cells and implications for cancer formation. Biochim Biophys Acta Mol Basis Dis. 2018;1864(1):89-101.
28. Ghezraoui H, Piganeau M, Renouf B, et al. Chromosomal translocations in human cells are generated by canonical nonhomologous end-joining. Mol Cell. 2014;55(6):829-842.
29. Manier S, Salem KZ, Park J, Landau DA, Getz G, Ghobrial IM. Genomic complexity of multiple myeloma and its clinical implications. Nat Rev Clin Oncol. 2016;14(2):100-113.
30. Kawamura K, Qi F, Meng Q, Hayashi I, Kobayashi J. Nucleolar protein nucleolin functions in replication stress-induced DNA damage responses. J Radiat Res. 2019;60(3):281-288.
31. Spies J, Lukas C, Somyajit K, Rask M-B, Lukas J, Neelsen KJ. 53BP1 nuclear bodies enforce replication timing at under-replicated DNA to limit heritable DNA damage. Nat Cell Biol. 2019;21(4):487-497.
32. Sallmyr A, Fan J, Rassool FV. Genomic instability in myeloid malignancies: Increased reactive oxygen species (ROS), DNA double strand breaks (DSBs) and error-prone repair. Cancer Lett. 2008;270(1):1-9.
33. Rassool FV, Gaymes TJ, Omidvar N, et al. Reactive oxygen species, DNA damage, and error-prone repair: A model for genomic instability with progression in myeloid leukemia? Cancer Res. 2007;67(18):8762-8771.
34. Kawano Y, Roccaro AM, Ghobrial IM, Azzi J. Multiple myeloma and the immune microenvironment. Curr Cancer Drug Targets. 2017;17(9):806-818.
35. Marchica V, Toscani D, Corcione A, et al. Bone marrow CX3CL1/

fractalkine is a new player of the pro-angiogenic microenvironment in multiple myeloma patients. Cancers (Basel). 2019;11(3):321.
36. Pilarski LM, Mant MJ, Belch AR. Drug resistance in multiple myeloma: novel therapeutic targets within the malignant clone. Leuk Lymphoma. 1999;32(3-4):199-210.
37. Issa ME, Takhsha FS, Chirumamilla CS, Perez-Novo C, Vanden Berghe W, Cuendet M. Epigenetic strategies to reverse drug resistance in heterogeneous multiple myeloma. Clin epigenetics. 2017;9:17.