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Research paper

A cycle involving HMGB1, IFN-γ and dendritic cells plays a putative role in anti-tumor immunity

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ABSTRACT

An important subset in regulating antitumor immunity is the maturation and accumulation of intratumor dendritic cells (DCs), inducing potent T cell cytotoxicity. In this study, we explored how the soluble abundant high-mobility group box 1 protein (HMGB1) affected DC activation and retention within lung cancers, and in which way the resultant interferon-γ (IFN-γ) further enhanced DC maturation and accumulation. It was discovered that HMGB1 was correlated with DC markers HLA-DR and CD86 in lung cancers at both mRNA and protein level. Further analyses showed HMGB1 enhanced the maturation of DCs, indicated by upregulated IFN-γ in $CD8^+$ T cells. Additionally, HMGB1 increased the accumulation of DCs by promoting CCR5 and CXCR3 production. Moreover, the resultant IFN-γ elevated the levels of HMGB1 and DC-associated chemokines, CCL5, CXCL10 and CXCL11 in tumor cells. Hence, the HMGB1-IFN-γ cycle may represent an important mechanism underlying DC-mediated anti-tumor immune response.

1. Introduction

Dendritic cells (DCs) are central players in anti-tumor immune response. In clinical trials, DC vaccinations have shown great success in treating tumors [1,2]. Mature DCs activate T lymphocytes by means of antigen-presentation, additionally, they evoke the persistent activation of T cells through CD80/CD86-CD28 interaction [3–5]. Local DCs are important in facilitating anti-tumor effects by interacting with intratumoral cytotoxic T lymphocytes (CTLs) [5,6]. In the absence of DCs, the proliferation of CTLs as well as their cytotoxic functions are compromised in malignancies. Additionally, the tumoral infiltration of T cells is hampered in the absence of DCs [7]. Despite the immune functions of DCs aforementioned, the mechanisms underlying DC maturation and accumulation within malignancies is not well understood.

Danger-associated molecular patterns (DAMPs) can induce both innate and adaptive immune responses [8]. Among DAMPs, the prototypic protein HMGB1 is well-characterized by promoting a local inflammatory response [9]. HMGB1 is released in response to cellular stress, causing it to bind with various receptors. Upon such binding, HMGB1 may modulate cell proliferation, differentiation, migration, gene expression, and cytokine release [6,10,11]. Moreover, HMGB1 is endowed with immuno-regulatory properties which trigger inflammatory and immune responses [12,13]. The enhanced release of HMGB1 synergizes cytotoxic effects of chemical drugs by inducing anticancer immune responses [14,15]. HMGB1 is able to promote expansion, survival, and cytokine production of $CD4^+$ and $CD8^+$ T cells [16–18]. This is probably related with the modulatory function of HMGB1 on DCs. However, it is unclear how HMGB1 regulates DCs

Abbreviations: IFN-γ, interferon-γ; HMGB1, high-mobility group box 1; DC, dendritic cell; CTL, cytotoxic T lymphocyte; DAMPs, danger-associated molecular patterns; PBMCs, peripheral blood mononuclear cells; TLR4, toll-liker receptor 4

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within the tumor microenvironment.

In this study, we explored the effect that HMGB1 has on DC maturation and retention in lung cancers as well as the relationship between DCs and $CD8^+$ T cell function and IFN-γ release. Furthermore, the effects of IFN-γ secretion on HMGB1 and DCs were also investigated.

2. Materials and methods

2.1. Clinical samples

The study was approved by the Ethics Committee Board of the First Affiliated Hospital of Zhengzhou University. Tissue samples were obtained from clinically diagnosed lung cancer patients and written informed consents were obtained. (REB tracking number research-2014-LW-23.)

2.2. Cell purification

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of healthy donors by Ficoll (Sigma-Aldrich, Saint Quentin-Fallavier, France) density gradient centrifugation. Then $CD14^+$ and $CD8^+$ cells were purified by using CD14- or CD8-isolating kit (Miltenyi Biotec, Auburn CA). The purity of isolated cells was confirmed using FACS assay.

2.3. DC induction

The $CD14^+$ cell fraction (> 93% purity) was cultured in a 6-well suspension culture plate for 5 days 1×10^6/mL in RPMI-1640 (Hyclone) medium supplemented with 10% FBS (Gibco) in the presence of 60 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ) and 70 ng/mL IL-4 (PeproTech, Rocky Hill, NJ) to achieve immature DCs [19]. After 5 days of culture, DCs were stimulated with 100 ng/mL HMGB1 (Biolegend, San Diego, CA) or the vehicle DMSO for an additional 24 h.

2.4. FACS assay

After 6 days of culture, the mature DCs were then collected and stained with flow antibodies [20]. Cells were counted, washed with PBS and incubated with antibodies for 15 min at 4 °C. Cells were washed again, resuspended and analyzed using a BD LSR II flow cytometer. Data were analyzed with CellQuest version 3.3 software (BD Biosciences). Surface antigen staining was performed using mouse antibodies against human CD14 (Cat. No.353706), CD83 (Cat. No. 305306), CD86 (Cat. No. 374204), HLA-DR (Cat. No. 307632) and human chemokine receptors CCR2 (Cat. No. 357216), CCR5 (Cat. No.313705), CCR6 (Cat. No.353412), CXCR2 (Cat. No. 320710), CXCR3 (Cat. No.305308), CXCR7 (Cat. No.331116) (BioLegend, Inc.). Appropriate fluorochrome-conjugated isotype control antibodies (BioLegend, Inc.) were used.

2.5. Chemotaxis assay

Lung cancer tissues were cut into small cubes and incubated in serum free cell culture media for 48 h. The culture supernatants were collected and added into the lower chambers of transwell plates in the absence or presence of CCR5 (ab201585) or CXCR3 (ab64714) neutralizing antibodies (Abcam, Cambridge, MA, USA). After 2 h incubation at 37 °C, the cells that migrated to the lower chambers of the transwell plate were counted.

2.6. RT-PCR and quantitative PCR

Total RNA was extracted with TRIzol reagent (TransGen) and reverse transcribed with a Reverse Transcription System (Promega).

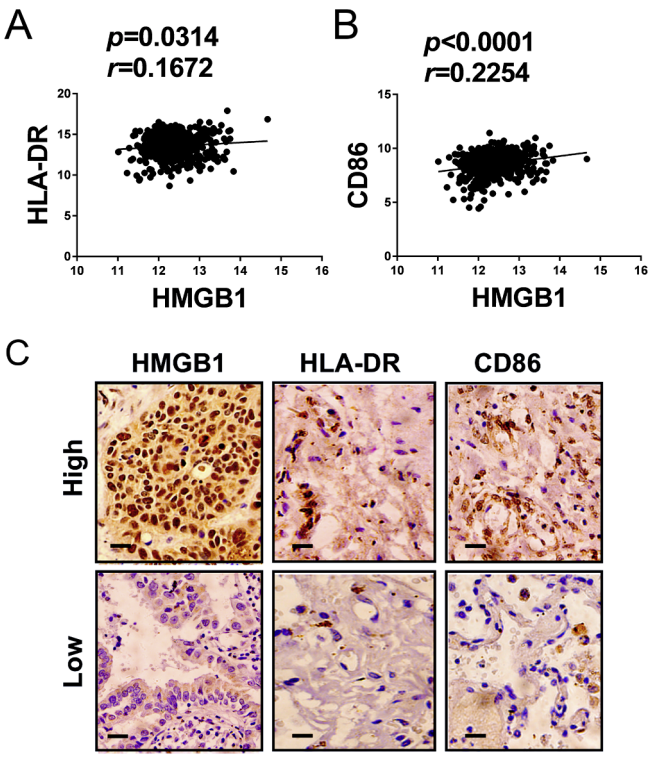

Fig. 1. HMGB1 correlates with DC maturation markers in lung cancers. (A, B) HMGB1 was positively related with DC markers, HLA-DR (A) and CD86 (B) based on the RNAseq data from TCGA network (n = 463) (http://cancergenome.nih.gov/). (C) Immunohistochemical analysis of lung cancer tissues for HMGB1, HLA-DR and CD86 (n = 50).

Table 1
Associations of HMGB1 with HLA-DR and CD86 in lung cancers (n = 50).

	HMGB1		p-value
	No. of weak staining (Percentage)	No. of strong staining (Percentage)	
HLA-DR No. of weak staining (Percentage)	24 (48%)	10 (20%)	0.0001
No. of strong staining (Percentage)	2 (4%)	14 (28%)	
CD86 No. of weak staining (Percentage)	19 (38%)	16 (32%)	0.0252
No. of strong staining (Percentage)	3 (6%)	12 (24%)	

P value < 0.05 indicated the correlation is significant.

Reverse-transcription products of different samples were amplified by a Light-Cycler System (Roche) using SYBR Green PCR mastermix (Applied Biosystems), according to the manufacturer's instructions. Data were normalized to the level of GAPDH expression in each individual sample. The 2-ΔΔCt method was used to calculate relative expression changes. The PCR products were visualized in agarose gel to avoid any primer dimer and nonspecific amplifications.

2.7. Multi-analyte flow assay

Tumor cells were seeded in 24-well plates at a density of 2×10^5 cells/well, and treated with 500 ng/ml IFN-γ (PeproTech) for 24 h. Then chemokine secretions were analyzed using the human pro-inflammatory chemokines detection kit according to manufacturer protocol (R&D Systems). In this assay, a total of 13 human chemokines

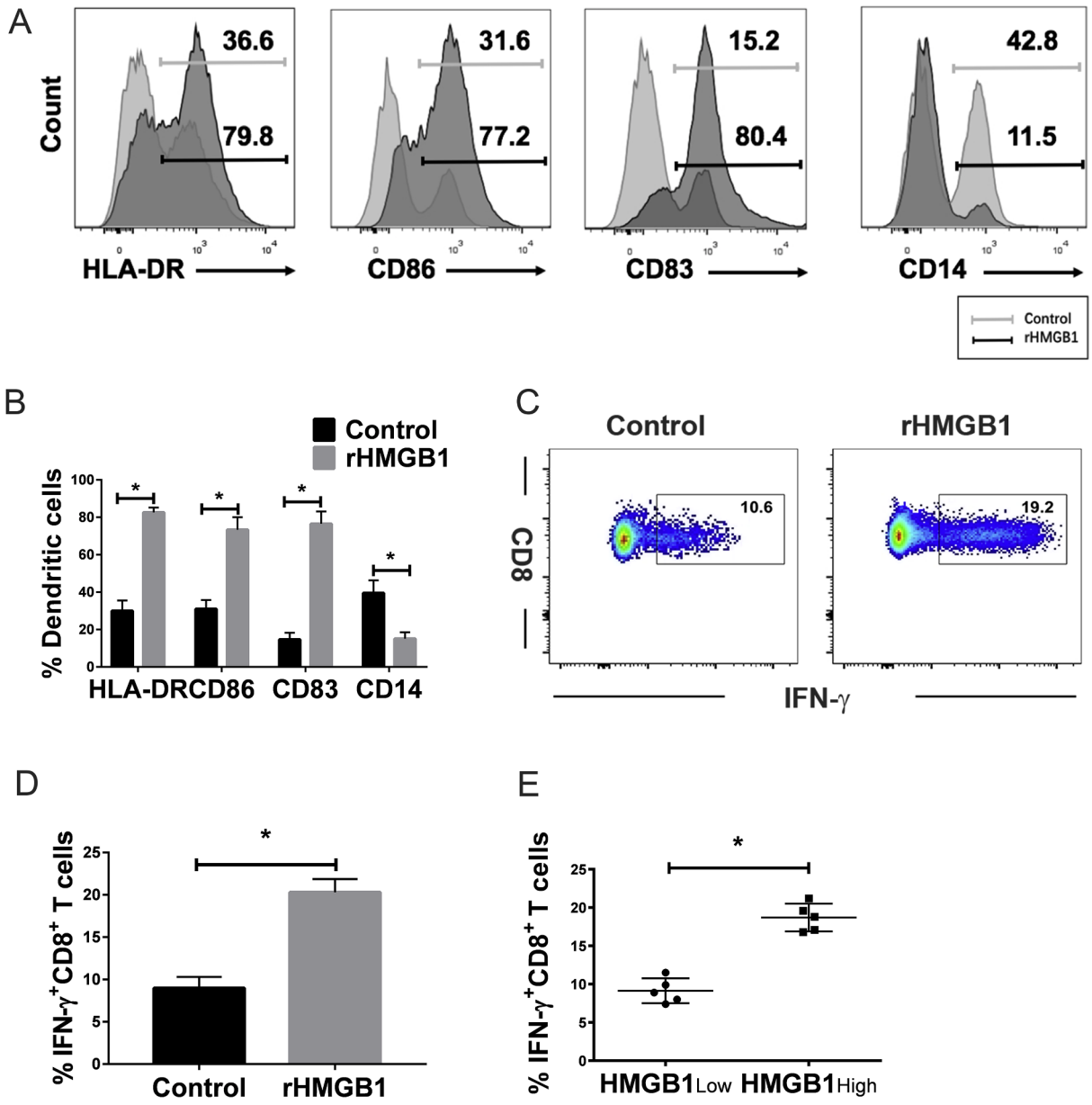

Fig. 2. HMGB1 enhances the maturation of DCs. (A, B) Compared to DMSO, HMGB1 promoted DC maturation. After the 5-day induction, DCs were treated with DMSO or HMGB1 for another 24 h. Then the expressions of HLA-DR, CD86, CD83 and CD14 were analyzed by flow cytometry. (C, D) Autologous $CD8^+$ T cells were co-cultured with antigen-loaded DCs for 48 h as described in materials and methods, and the intracellular expressions of IFN-γ in T cells were monitored by flow cytometry. (E) The portions of IFN-γ^+CD8^+ T cells were analyzed in primary tumors with high levels of HMGB1 ($HMGB1_{High}$) or low levels ($HMGB1_{Low}$). *, $P < 0.05$.

were detected, including MCP-1 (CCL2), RNATES (CCL5), IP-10 (CXCL10), Eotaxin (CCL11), TARC (CCL17), MIP-1α (CCL3), MIP-1β (CCL4), MIG (CXCL9), MIP-3α (CCL20), ENA78 (CXCL5), GROα (CXCL1), I-TAC (CXCL11) and IL-8 (CXCL8).

2.8. Immunohistochemistry assay

Formalin-fixed, paraffin-embedded sections (3 μm) were baked at 60 °C for 1 h, deparaffinized in xylene, then rehydrated through graded alcohol and washed briefly in tap water. Endogenous peroxidase was blocked by incubation in methanol containing 0.3% hydrogen peroxide for 30 min. To retrieve antigenicity, sections were boiled in 10 mmol/L citrate buffer (pH 5.8) for 30 min in microwave (800 W). Sections were then incubated with goat serum diluted in PBS (pH 7.4) at room temperature for 30 min. Subsequently, sections were incubated at 4 °C overnight with the primary antibodies specific for IFN-γ (ab218426), HMGB1 (ab77302), CXCL10 (ab9807), CXCL11 (ab9955), CCL5 (ab189841), HLA-DR (ab175085) or CD86 (ab53004) (Abcam, Cambridge, MA, USA). Sections were rinsed with fresh PBS and incubated with horseradish peroxidase-linked secondary antibodies at room temperature for 30 min. Lastly, sections were stained with DAB substrate (Dako, Carpinteria, CA, USA) and counterstained with Mayer's hematoxylin. Photos were recorded under microscopy (Leica, Wetzlar, Germany). The expressions of interested genes were evaluated based on the intensity (none = 0, low = 1, moderate = 2 and high = 3) of protein staining and the density (0% = 0, 1–40% = 1, 41–75% = 2 and > 76% = 3) of positive cells. The scores of sections were multiplied intensity and density. The samples scored 5 or over were regarded as strong expression.

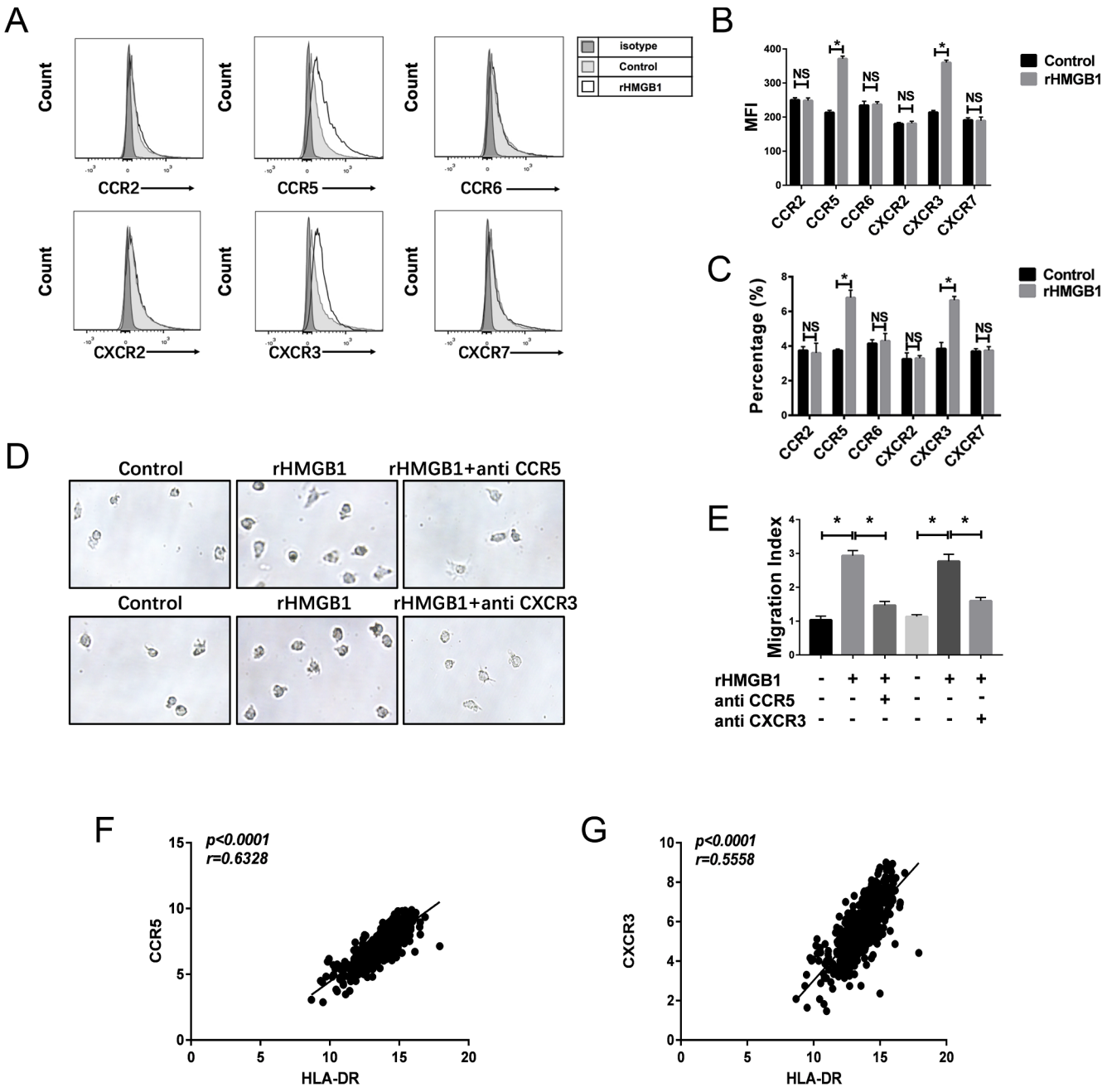

Fig. 3. HMGB1 upregulates CCR5 and CXCR3 on DCs. (A, B, C) With HMGB1 treatment, the surface expressions of CCR5 and CXCR3 in DCs were expanded as determined by flow cytometer. (D, E) When adding tumor culture supernatants in lower wells, the directional movements of DCs were enhanced after HMGB1-incubation, but reversed by CCR5- or CXCR3-neutralizing antibodies. *$P < 0.05$. (F, G) Data from TCGA indicated HLA-DR is positively related with CCR5 and CXCR3 (n = 463).

2.9. Co-culture assay

Tumor tissues were fractured, freezed and thawed for 3 cycles. Then tumor lysates were collected and protein concentrations were determined. Following the 6-day DC induction, autologous DCs were inoculated with tumor lysates for 24 h. Following which, autologous $CD8^+$ T cells were primed with the antigen-loaded DCs for an additional 2-day period with 100 IU/mL IL-2. The $CD8^+$ T cells were analyzed for intracellular IFN-γ after the 2-day co-culture period.

2.10. Statistical analysis

The data shown represents at least 3 independent experiments. One-way ANOVA and T test were performed to compare the intra- and inter-group differences. The Regression test was used to determine the correlation of genes. RNAseq data from The Cancer Genome Atlas (TCGA) network (n = 463) (http://cancergenome.nih.gov/) were converted by formula [f(x) = log2] in excel. The p values < 0.05 were considered as statistically significant.

3. Results

3.1. HMGB1 is related with DC maturation markers in lung cancers

Through the biocomputation approach, the correlations of HMGB1 was confirmed with HLA-DR and CD86, which mainly expressed on

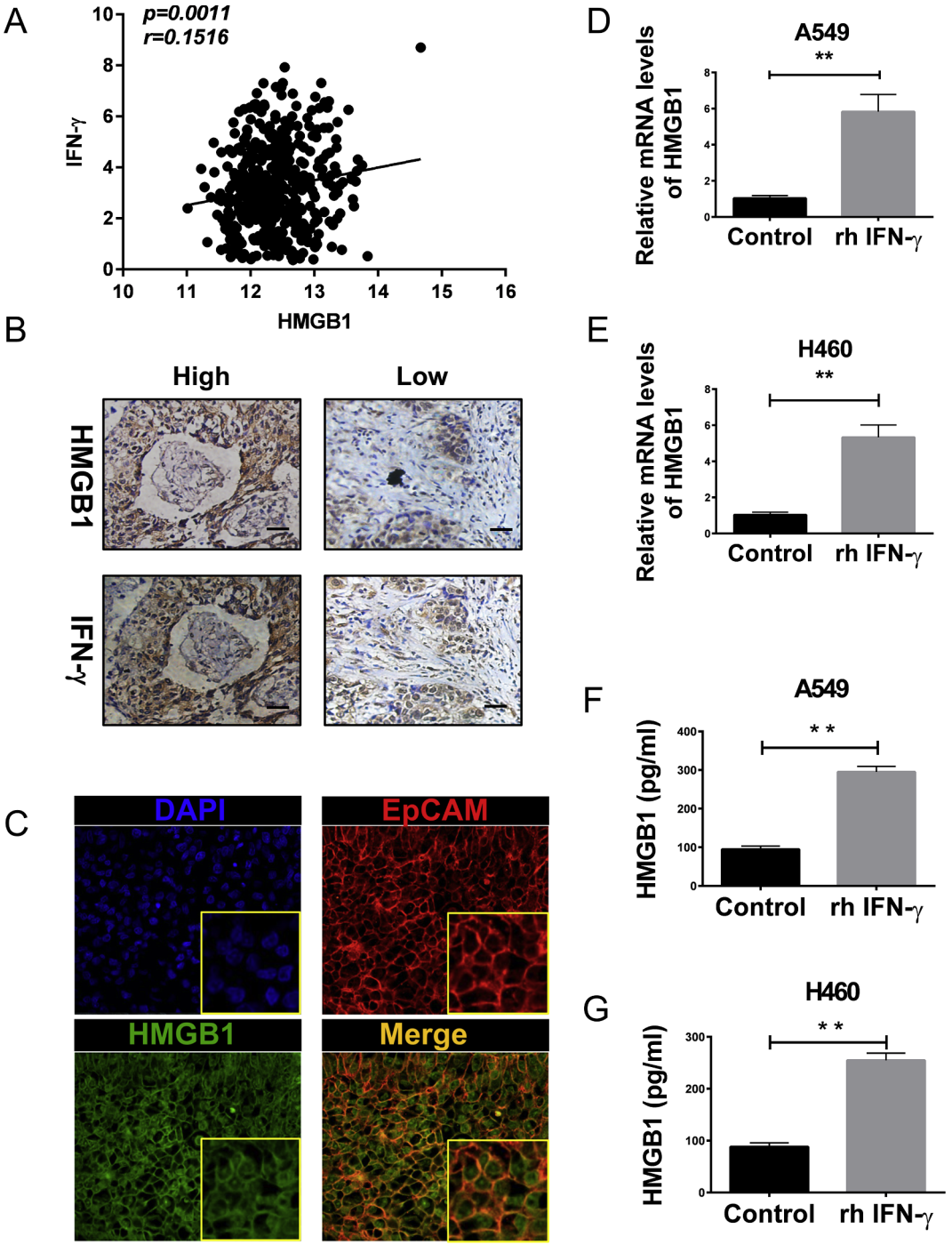

Fig. 4. IFN-γ increases HMGB1 release. (A) Correlation analysis of HMGB1 and IFN-γ expressions in lung cancer specimens from TCGA database (n = 463). (B) IHC staining of HMGB1 and IFN-γ in lung cancer tissues using serial sections. (C) Immunofluorescence assay of tumor sections showing malignant cells ($CD326^+$) were the major source of HMGB1. (D, E) HMGB1 expressions were determined using RT-PCR in A549 and H460 lung cancer cells conditioned with IFN-γ for 24 h. (F, G) A549 and H460 cells were treated with IFN-γ for 24 h, and supernatants were collected to run ELISA test for soluble HMGB1. $^{**}P < 0.01$.

mature DCs. We analyzed HMGB1, HLA-DR and CD86 mRNA levels of 463 NSCLC patients from TCGA database. As showed in Fig. 1A and B, HLA-DR and CD86 demonstrated positive correlations with HMGB1 despite the coefficient was not very high ($r_{HLA\text{-}DR} = 0.1672$, $p = 0.0314$; $r_{CD86} = 0.2254$, $p < 0.0001$). Next, we checked this correlation in samples from malignant tissues of 50 clinically diagnosed lung cancer patients. To validate this association in primary tumor tissues, we investigated the expression of HMGB1, HLA-DR and CD86 by using IHC (Fig. 1C). As expected, the correlations of HMGB1 with the markers of DC maturation, HLA-DR and CD86, were positive at protein level as well ($p_{HLA\text{-}DR} = 0.0001$; $p_{CD86} = 0.0252$) (Table 1), indicating that HMGB1 possibly promotes either accumulation or maturation of DCs.

3.2. HMGB1 enhances the maturation of DCs

To investigate how HMGB1 affects DCs in cancerous lesions, we first investigated whether or not HMGB1 promoted the maturation of DCs. It was found that HMGB1 resulted in the upregulation of DC related genes. At first, we checked the surface expressions of HLA-DR, CD83

Table 2
Preferential expressions of HMGB1 with IFN-γ in lung cancers (n = 50).

	IFN-γ		p-value
	No. of weak staining (Percentage)	No. of strong staining (Percentage)	
HMGB1 No. of weak staining (Percentage)	23 (46%)	15 (30%)	0.0081
No. of strong staining (Percentage)	2 (4%)	10 (20%)	

P value < 0.05 was considered with significant correlation.

and CD86, whose intensities demonstrate the activation of DC [21]. $CD14^+$ cells were cultured with GM-CSF and IL-4 for 5 days so as to yield immature DCs. Then HMGB1 was added to induce DC activation. Relative to the control, treated with DMSO, DCs treated with HMGB1 demonstrated higher expressions of HLA-DR, CD86 and CD83, and decreased CD14 (Fig. 2A and B). We observed T cell function after they had been co-cultured with HMGB1-treated DC, confirming that mature DCs facilitate T cell activation. As presented in Fig. 2C and D, there was a high number of INF-γ positive $CD8^+$ T cells that had been co-inoculated with HMGB1-primed DCs, confirming that HMGB1 enhances DC maturation. We examined the effect of HMGB1 on perforin and Granzyme B in $CD8^+$ T cells, the results showed that HMGB1 can upregulate the expression of both effector molecules (Fig. S1). Consistently, HMGB1 could enhance T cell activation and IFN-γ production by augmenting the function of DCs in cancer patients. According to the expression level of HMGB1, the patients were divided into HMGB1 high and low expression groups. We found that infiltrated $CD8^+$ T cells had greater portions of IFN-γ positive subsets in cancer patients with increased intratumor expression of HMGB1 (Fig. 2E).

3.3. HMGB1 increases CCR5 and CXCR3 on DCs

Recruitment and retention of DCs into malignant tissues, impact on the intratumoral accumulation. We explored the expressions of chemokine receptors on DCs, which are closely associated with the gathering of immune cells. After HMGB1 treatment, CXCR3 and CCR5 were upregulated on DCs (Fig. 3A–C). In order to further verify these results, we used Western blot to detect the effect of HMGB1 on the expression of CCR5 and CXCR3, and equally the results showed that HMGB1 significantly up-regulated the expression of CCR5 and CXCR3 (Fig. S2). We further found that HMGB1 did not directly affect the expression of receptors on T cells (Fig. S3). DC migration was investigated using the tumor tissue culture supernatants. The directional movement was enhanced in HMGB1-treated DCs (Fig. 3D and E). This enhanced migration was reversed by CCR5- or CXCR3-neutralizing antibodies (Fig. 3D and E). Besides, those 2 chemokine receptors showed strong relations with DC marker, HLA-DR ($r_{CCR5} = 0.6328$, $p < 0.0001$; $r_{CXCR3} = 0.5558$, $p < 0.0001$) (Fig. 3F and G). However, HMGB1 did not alter the CCR5 ligand (CCL5) and CXCR3 ligands (CXCL10 and CXCL11) levels in DCs (Fig. S4A), and the expressions of CCL5, CXCL10 and CXCL11 in tumor cells were significantly higher than that in DCs (Fig. S4B). In addition to enhancing the maturation of DCs, these data suggest that HMGB1 enhances the migration of DCs.

3.4. IFN-γ is positively associated with HMGB1

As aforementioned, HMGB1 increases IFN-γ production by promoting DC-induced T cell activation. Reciprocally, IFN-γ increases the release of DAMP molecules including HMGB1. To confirm this hypothesis, we monitored the relationship between IFN-γ and HMGB1. We analyzed the HMGB1 and IFN-γ mRNA levels of 463 NSCLC patients from TCGA database. And the results showed the correlation of IFN-γ with HMGB1 was mildly but significantly positive ($r = 0.1516$, $p = 0.0011$) (Fig. 4A). Additionally, IHC assay supported that IFN-γ had a positive relationship with HMGB1 ($p = 0.0081$) (Fig. 4B; Table 2). The upregulation of HMGB1 seemed IFN-γ-dependent, this was shown in the analysis of the multiplex genes in the reflected tumors. Among the common cytokines, only IFN-γ showed a similar trend to HMGB1 (Fig. S5A).

Tests were conducted to ascertain IFN-γ induced HMGB1 production. The main source of HMGB1 in tumor tissues was determined using the IF technique. As shown in Figs. 4C and S6, $CD326^+$ ($EPCAM^+$) cancer cells were the major cell type expressing HMGB1. The production of HMGB1 in IFN-γ-stimulated lung cancer cells was observed. As expected, the transcription of HMGB1 was upregulated in 2 lung cancer cell lines, A549 and NCI-H460 ($p < 0.01$; $p < 0.01$) (Fig. 4D and E). Consistently, the translation and secretion of HMGB1 were increased in IFN-γ-stimulated lung cancer cells ($p < 0.01$; $p < 0.01$) (Figs. 4F and G; S5B).

3.5. IFN-γ stimulates tumor cells to secrete CCL5, CXCL10 and CXCL11

In addition to upregulate HMGB1, IFN-γ modulated chemokines expression (Fig. 5A). The multi-analyte flow assay showed that CCL5, CXCL10 and CXCL11 were increased in IFN-γ-treated A549 cells (Fig. 5A). This finding was further confirmed in A549 and NCI-H460 cells using qRT-PCR (A549: $p_{CCL5} < 0.05$; $p_{CXCL10} < 0.01$; $p_{CXCL11} < 0.01$; NCI-H460: $p_{CCL5} < 0.05$; $p_{CXCL10} < 0.01$; $p_{CXCL11} < 0.05$) (Fig. 5B). Consistently, IFN-γ enhanced the expressions of CCL5, CXCL10 and CXCL11, which are recognized by CCR5 and CXCR3. Intriguingly, CCR5 and CXCR3 were upregulated by HMGB1 treated DCs (Fig. 3A–C). TCGA data also supported that IFN-γ was positively related with those specific chemokines ($r_{CCL5} = 0.6910$, $p < 0.0001$; $r_{CXCL10} = 0.4256$, $p < 0.0001$; $r_{CXCL11} = 0.5028$, $p < 0.0001$) (Fig. 5C). Moreover, IHC analysis confirmed that intratumoral IFN-γ densities were tightly associated with CCL5, CXCL10 and CXCL11 production within lung cancer cells ($p = 0.0004$; $p = 0.0055$; $p = 0.0129$) (Fig. 5D; Table 3).

4. Discussion

The tumor microenvironment consists of tumor cells, immune cells, stromal cells and soluble factors. These components interact with each other to regulate an anti-tumor immune response. In this study, we found that the soluble factor HMGB1 enhanced the maturation and accumulation of DCs in lung cancer. HMGB1 also promoted $CD8^+$ T cell activation and facilitated the upregulation of IFN-γ. IFN-γ further enhanced HMGB1 secretion from tumor cells, which may eventually increase functional DCs. We propose that the DC-mediated HMGB1-IFN-γ loop in Fig. 6, could represent a putative regulatory mechanism in the anti-tumor immune response.

DCs are specialized in initiating *T*-cell immunity. Mature DCs have the ability to activate $CD8^+$ cytotoxic T lymphocytes and therefore play an essential role in anti-tumor immune responses [22,23]. However, DCs are frequently ineffective at presenting their antigens to T cells because immature DCs are sustained by tumor-derived immunosuppressive factors [24,25]. We found that HMGB1, a factor mainly released by tumor cells, increased the maturation of DCs. This was consistently confirmed in 2 previous studies [26,27]. During cellular stress, HMGB1 is released from tumor cells, this induces DC maturation by binding with toll-like receptor 4 (TLR4), the receptor involved in DC antigen presentation [28]. Indeed, our data showed that HMGB1 was positively correlated with HLA-DR and CD86, which present on mature DCs in lung cancers (Fig. 1), indicating that HMGB1 possibly promotes either accumulation or maturation of DCs. *In vitro* experiments confirmed that HMGB1 promotes the activation of DCs. Upon HMGB1 stimulation, DCs exhibited stronger functions as

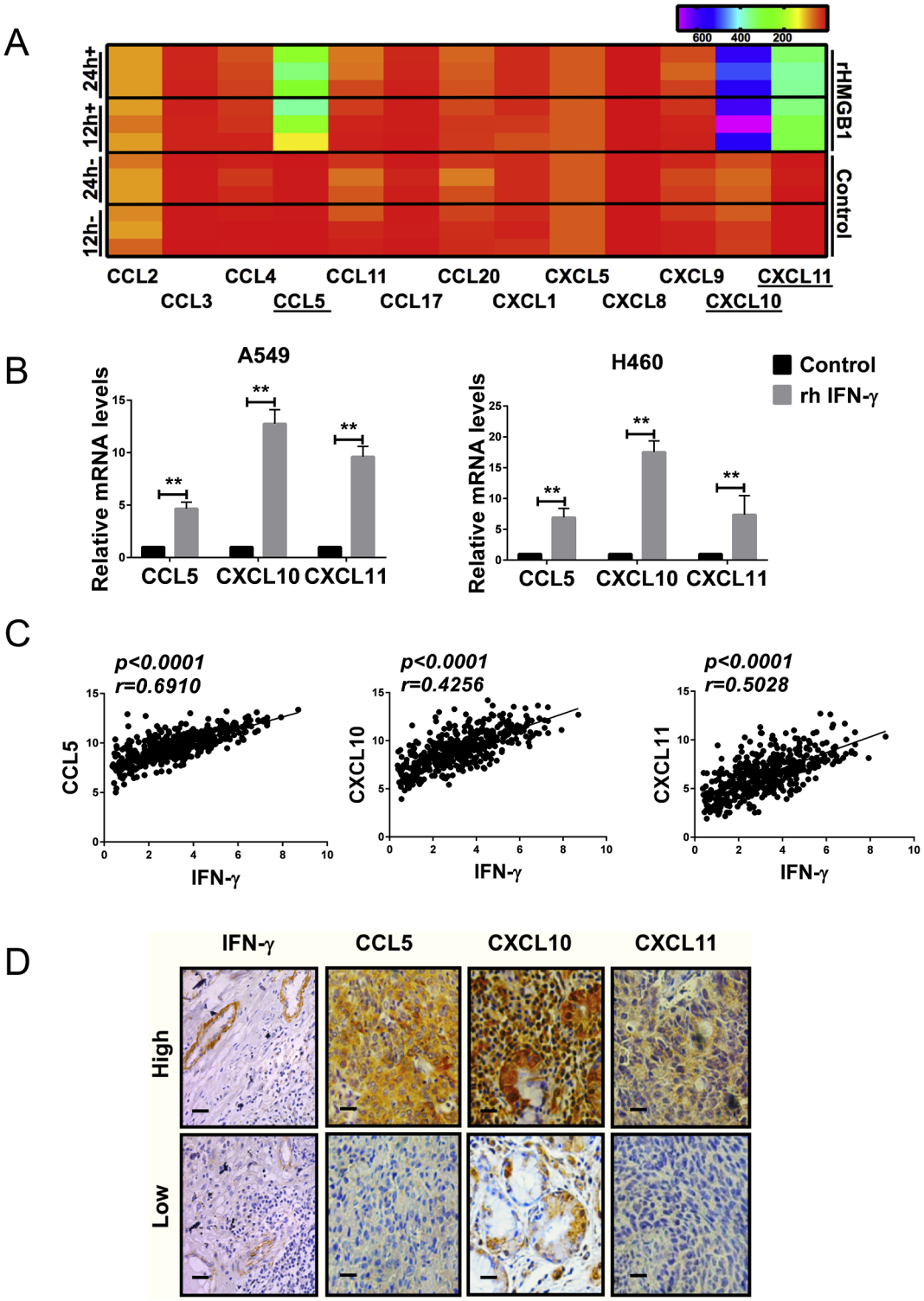

Fig. 5. IFN-γ upregulates specific chemokines expression. (A) Multi-Analyte Assay of 13 chemokines expression in A549 cells after IFN-γ treatment. (B) Upregulations of CCL5, CXCL10 and CXCL11 by IFN-γ in A549 and NCI-H460 cells were confirmed by RT-PCR. **, $P < 0.01$. (C) The correlations of IFN-γ with CCL5, CXCL10 and CXCL11 based on TCGA database (n = 463). (D) IHC studied on tumor tissue sections for IFN-γ, CCL5, CXCL10 and CXCL11.

indicated by the upregulation of activation markers, HLA-DR, CD83 and CD86 (Fig. 2A and B). As the sequence, DC-directed activation of $CD8^+$ T cells was obviously enhanced (Fig. 2C and D). Moreover, in those clinical samples expressing high levels of HMGB1 and hence having more mature DCs, IFN-γ-producing $CD8^+$ T cells were more frequent (Fig. 2E; Tables 1 and 2). Together with others' reports, it is believing that HMGB1 is critical for DC maturation and subsequent T cell activation.

Besides, HMGB1 also modulates chemokine receptors on DCs. DCs exhibit features of mobile cells, and their intratumoral distribution is crucial for their action *in vivo*. Previous studies have shown that chemokines and their respective receptors are vital for the directional migration and tissue-residence of DCs [29,30]. In this report, we noticed that HMGB1 increased CCR5 and CXCR3, whose ligand was

Table 3
IFN-γ is related with CCL5, CXCL10 and CXCL11 in lung cancers (n = 50).

	IFN-γ		p-value
	No. of weak staining (Percentage)	No. of strong staining (Percentage)	
CCL5 No. of weak staining (Percentage)	25 (50%)	11 (22%)	0.0004
No. of strong staining (Percentage)	2 (4%)	12 (24%)	
CXCL10 No. of weak staining (Percentage)	22 (44%)	13 (26%)	0.0055
No. of strong staining (Percentage)	3 (6%)	12 (24%)	
CXCL11 No. of weak staining (Percentage)	20 (40%)	13 (26%)	0.0129
No. of strong staining (Percentage)	4 (8%)	13 (26%)	

P value < 0.05 indicated the relation is significant.

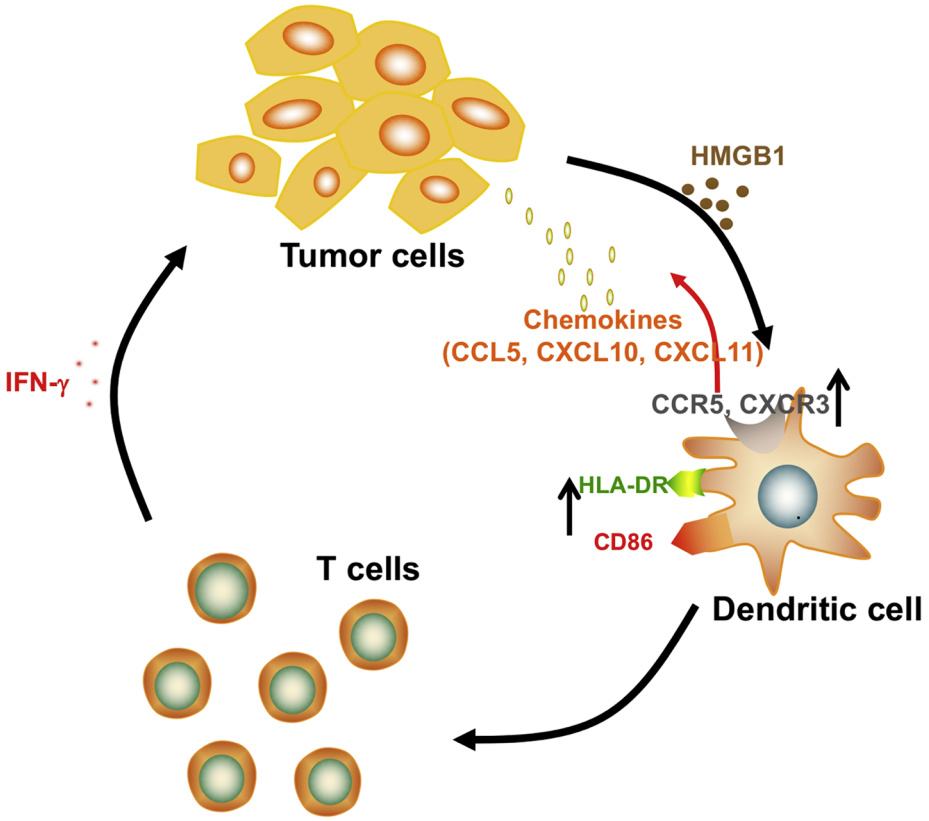

Fig. 6. Schematic diagram showing the positive feedback loop of HMGB1- IFN-γ mediated by DCs.

upregulated by IFN-γ (Figs. 3A–C; 5A and B). Additionally, CCR5 and CXCR3 enhanced the migration and retention of DCs in lung cancer (Fig. 3D–G). Furthermore, the cognate ligands CCL5, CXCL10 and CXCL11 were analyzed for their correlations with DCs, instead of the receptors expressed on various types of cells. In consistent with the findings above, CCL5, CXCL10 and CXCL11 were positively related with HLA-DR ($r_{HLA\text{-}DR/CCL5} = 0.4796$, $p < 0.0001$; $r_{HLA\text{-}DR/CXCL10} = 0.3840$, $p < 0.0001$; $r_{HLA\text{-}DR/CXCL11} = 0.4492$, $p < 0.0001$) (Fig. S7). In addition to directly enhancing the activation of DCs, HMGB1 further augmented antigen-presentation by increasing DC accumulation. This modulatory effect on chemokine receptors of HMGB1 may be attributed to the activation of TLR4-NFκB signal [31–33]. However, the specific underlying mechanism should be explored in future studies.

Mature DCs are able to drive T cell polarization and activation, as evidenced by IFN-γ secretion [27]. Consistently, HMGB1 could enhance T cell activation and IFN-γ production by augmenting the function of DCs (Fig. 2D and E). As the inducer of DAMP molecules, IFN-γ tuned up the secretion of HMGB1 (Fig. 4D–G), and this effect was likely IFN-γ-specific. In lung cancer samples, we detected HMGB1 and other abundant pro-inflammatory and immunosuppressive cytokines. Only IFN-γ showed concomitant changes with HMGB1 at mRNA level (Fig. S5A). HMGB1 was able to promote DC activation and IFN-γ was found to further enhance DC maturation by upregulating HMGB1. Moreover, IFN-γ increased the expressions of DC-associated chemokines (Fig. 5). Together with their receptor, CCR5 and CXCR3 that were upregulated by HMGB1 (Fig. 3), those IFN-γ-induced chemokines would strengthen the retention and accumulation of DCs in malignant lesions. The correlation analysis indicated a strong connection between certain chemokines and DCs (Fig. S7). These observations suggested that IFN-γ is not only important in DC maturation, but also enhancing DC function by increasing DC accumulation and maturation in tumors. Indeed, IFN-γ exhibited biased expression with DCs (Table S1), indicating the partnership between this proinflammatory cytokine and the tumor-sensing cells.

The tumor microenvironment usually decreases the functions of DCs by compromising their maturation as well as their recruitment, thus escaping immune-surveillance. In this report, we uncovered an important role of HMGB1 and IFN-γ in enhancing DC function in lung

cancers. IFN-γ-upregulated HMGB1 facilitated the activation and accumulation of DCs, which finally promoted IFN-γ production. Hence, this cycle involving HMGB1, IFN-γ and DCs plays pivotal roles in anti-tumor immunity.

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Declarations of conflict of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cellimm.2018.08.011.

References

[1] P.A. Ott, Z. Hu, D.B. Keskin, S.A. Shukla, J. Sun, D.J. Bozym, W. Zhang, A. Luoma, A. Giobbie-Hurder, L. Peter, C. Chen, O. Olive, T.A. Carter, S. Li, D.J. Lieb, T. Eisenhaure, E. Gjini, J. Stevens, W.J. Lane, I. Javeri, K. Nellaiappan, A.M. Salazar, H. Daley, M. Seaman, E.I. Buchbinder, C.H. Yoon, M. Harden, N. Lennon, S. Gabriel, S.J. Rodig, D.H. Barouch, J.C. Aster, G. Getz, K. Wucherpfennig, D. Neuberg, J. Ritz, E.S. Lander, E.F. Fritsch, N. Hacohen, C.J. Wu, An immunogenic personal neoantigen vaccine for patients with melanoma, Nature 547 (2017) 217–221.

[2] U. Sahin, E. Derhovanessian, M. Miller, B.P. Kloke, P. Simon, M. Lower, V. Bukur, A.D. Tadmor, U. Luxemburger, B. Schrors, T. Omokoko, M. Vormehr, C. Albrecht, A. Paruzynski, A.N. Kuhn, J. Buck, S. Heesch, K.H. Schreeb, F. Muller, I. Ortseifer, I. Vogler, E. Godehardt, S. Attig, R. Rae, A. Breitkreuz, C. Tolliver, M. Suchan, G. Martic, A. Hohberger, P. Sorn, J. Diekmann, J. Ciesla, O. Waksmann, A.K. Bruck, M. Witt, M. Zillgen, A. Rothermel, B. Kasemann, D. Langer, S. Bolte, M. Diken, S. Kreiter, R. Nemecek, C. Gebhardt, S. Grabbe, C. Holler, J. Utikal, C. Huber, C. Loquai, O. Tureci, Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer, Nature 547 (2017) 222–226.

[3] M.L. Broz, M. Binnewies, B. Boldajipour, A.E. Nelson, J.L. Pollack, D.J. Erle, A. Barczak, M.D. Rosenblum, A. Daud, D.L. Barber, S. Amigorena, L.J. Van't Veer, A.I. Sperling, D.M. Wolf, M.F. Krummel, Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity, Cancer Cell 26 (2014) 638–652.

[4] A. Schietinger, M. Philip, V.E. Krisnawan, E.Y. Chiu, J.J. Delrow, R.S. Basom, P. Lauer, D.G. Brockstedt, S.E. Knoblaugh, G.J. Hammerling, T.D. Schell, N. Garbi, P.D. Greenberg, Tumor-specific T cell dysfunction is a dynamic antigen-driven differentiation program initiated early during tumorigenesis, Immunity 45 (2016) 389–401.

[5] J.J. Engelhardt, B. Boldajipour, P. Beemiller, P. Pandurangi, C. Sorensen, Z. Werb, M. Egeblad, M.F. Krummel, Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific T cells, Cancer Cell 21 (2012) 402–417.

[6] T.S. Kim, S.A. Gorski, S. Hahn, K.M. Murphy, T.J. Braciale, Distinct dendritic cell subsets dictate the fate decision between effector and memory CD8(+) T cell differentiation by a CD24-dependent mechanism, Immunity 40 (2014) 400–413.

[7] S. Spranger, D. Dai, B. Horton, T.F. Gajewski, Tumor-residing Batf3 dendritic cells are required for effector T cell trafficking and adoptive T cell therapy, Cancer Cell 31 (2017) 711–723.

[8] A.E.I.P.A.M. Uguccioni, Modulation of chemokine responses: synergy and cooperativity, Front. Immunol. 7 (2016) 1–6.

[9] P.R. do Nascimento, D.R. Martins, G.R. Monteiro, P.V. Queiroz, F.P. Freire-Neto, J.W. Queiroz, A.L. Morais Lima, S.M. Jeronimo, Association of pro-inflammatory cytokines and iron regulatory protein 2 (IRP2) with Leishmania burden in canine visceral leishmaniasis, PLoS One 8 (2013) 1–10.

[10] X. Qiang, W.L. Yang, R. Wu, M. Zhou, A. Jacob, W. Dong, M. Kuncewitch, Y. Ji, H. Yang, H. Wang, J. Fujita, J. Nicastro, G.F. Coppa, K.J. Tracey, P. Wang, Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis, Nat. Med. 19 (2013) 1489–1495.

[11] J.S. Dolina, T.J. Braciale, Y.S. Hahn, Liver-primed CD8+ T cells suppress antiviral adaptive immunity through galectin-9-independent T-cell immunoglobulin and mucin 3 engagement of high-mobility group box 1 in mice, Hepatology 59 (2014) 1351–1365.

[12] M.T. Lotze, K.J. Tracey, High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal, Nat. Rev. Immunol. 5 (2005) 331–342.

[13] M.T. Lotze, H.J. Zeh, A. Rubartelli, L.J. Sparvero, A.A. Amoscato, N.R. Washburn, M.E. Devera, X. Liang, M. Tör, T. Billiar, The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity, Immunol. Rev. 220 (2007) 60–81.

[14] D.V. Krysko, A.D. Garg, A. Kaczmarek, O. Krysko, P. Agostinis, P. Vandenabeele, Immunogenic cell death and DAMPs in cancer therapy, Nat. Rev. Cancer 12 (2012) 860–875.

[15] J.W. Hodge, C.T. Garnett, B. Farsaci, C. Palena, K.Y. Tsang, S. Ferrone, S.R. Gameiro, Chemotherapy-induced immunogenic modulation of tumor cells enhances killing by cytotoxic T lymphocytes and is distinct from immunogenic cell death, Int. J. Cancer 133 (2013) 624–636.

[16] I.E. Dumitriu, P. Baruah, B. Valentinis, R.E. Voll, M. Herrmann, P.P. Nawroth, B. Arnold, M.E. Bianchi, A.A. Manfredi, P. Rovere-Querini, Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products, J. Immunol. 174 (2005) 7506–7515.

[17] S. Jube, Z.S. Rivera, M.E. Bianchi, A. Powers, E. Wang, I. Pagano, H.I. Pass, G. Gaudino, M. Carbone, H. Yang, Cancer cell secretion of the DAMP protein HMGB1 supports progression in malignant mesothelioma, Cancer Res. 72 (2012) 3290–3301.

[18] E. Sundberg, A.E.R. Fasth, K. Palmblad, H.E. Harris, U. Andersson, High mobility group box chromosomal protein 1 acts as a proliferation signal for activated T lymphocytes, Immunobiology 214 (2009) 303–309.

[19] Z. Shao, R. Gaurav, D.K. Agrawal, Intermediate-conductance calcium-activated potassium channel KCa3.1 and chloride channel modulate chemokine ligand (CCL19/CCL21)-induced migration of dendritic cells, Transl. Res. 166 (2015) 89–102.

[20] J.Y. Liu, F. Li, L.P. Wang, X.F. Chen, D. Wang, L. Cao, Y. Ping, S. Zhao, B. Li, S.H. Thorne, B. Zhang, P. Kalinski, Y. Zhang, CTL- vs Treg lymphocyte-attracting chemokines, CCL4 and CCL20, are strong reciprocal predictive markers for survival of patients with oesophageal squamous cell carcinoma, Br. J. Cancer 113 (2015) 747–755.

[21] J. Fucikova, I. Moserova, I. Truxova, I. Hermanova, I. Vancurova, S. Partlova, A. Fialova, L. Sojka, P.F. Cartron, M. Houska, L. Rob, J. Bartunkova, R. Spisek, High hydrostatic pressure induces immunogenic cell death in human tumor cells, Int. J. Cancer 135 (2014) 1165–1177.

[22] Q. Fu, Y. Wu, F. Yan, N. Wang, W. Wang, X. Cao, Y. Wang, T. Wan, Efficient induction of a Her2-specific anti-tumor response by dendritic cells pulsed with a Hsp70L1-Her2(341–456) fusion protein, Cell. Mol. Immunol. 8 (2011) 424–432.

[23] K. Palucka, J. Banchereau, Cancer immunotherapy via dendritic cells, Nat. Rev. Cancer 12 (2012) 265–277.

[24] G.V. Shurin, Y. Ma, M.R. Shurin, Immunosuppressive mechanisms of regulatory dendritic cells in cancer, Cancer Microenviron. 6 (2013) 159–167.

[25] S. Demoulin, M. Herfs, P. Delvenne, P. Hubert, Tumor microenvironment converts plasmacytoid dendritic cells into immunosuppressive/tolerogenic cells: insight into the molecular mechanisms, J. Leukoc Biol. 93 (2013) 343–352.

[26] P. Rovere-Querini, A. Capobianco, P. Scaffidi, B. Valentinis, F. Catalanotti, M. Giazzon, I.E. Dumitriu, S. Muller, M. Iannacone, C. Traversari, M.E. Bianchi, A.A. Manfredi, HMGB1 is an endogenous immune adjuvant released by necrotic cells, EMBO Rep. 5 (2004) 825–830.

[27] D. Messmer, H. Yang, G. Telusma, F. Knoll, J. Li, B. Messmer, K.J. Tracey, N. Chiorazzi, High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization, J. Immunol. 173 (2004) 307–313.

[28] L. Apetoh, F. Ghiringhelli, A. Tesniere, M. Obeid, C. Ortiz, A. Criollo, G. Mignot, M.C. Maiuri, E. Ullrich, P. Saulnier, H. Yang, S. Amigorena, B. Ryffel, F.J. Barrat, P. Saftig, F. Levi, R. Lidereau, C. Nogues, J.P. Mira, A. Chompret, V. Joulin, F. Clavel-Chapelon, J. Bourhis, F. Andre, S. Delaloge, T. Tursz, G. Kroemer, L. Zitvogel, Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy, Nat. Med. 13 (2007) 1050–1059.

[29] T.H. Lo, P.A. Silveira, P.D. Fromm, N.D. Verma, P.A. Vu, F. Kupresanin, R. Adam, M. Kato, V.C. Cogger, G.J. Clark, D.N. Hart, Characterization of the expression and function of the C-type lectin receptor CD302 in mice and humans reveals a role in dendritic cell migration, J. Immunol. 197 (2016) 885–898.

[30] A.M. Schulz, S. Stutte, S. Hogl, N. Luckashenak, D. Dudziak, C. Leroy, I. Forne, A. Imhof, S.A. Muller, C.H. Brakebusch, S.F. Lichtenthaler, T. Brocker, Cdc42-dependent actin dynamics controls maturation and secretory activity of dendritic cells, J. Cell. Biol. 211 (2015) 553–567.

[31] D. Mitchell, C. Olive, Regulation of toll-like receptor-induced chemokine production in murine dendritic cells by mitogen-activated protein kinases, Mol. Immunol. 47 (2010) 2065–2073.

[32] J. Mahmoudi, B. Sabermarouf, B. Baradaran, L. Sadat-Hatamnezhad, S.S. Shotorbani, Up-regulation of TLR2 and TLR4 in high mobility group Box1-stimulated macrophages in pulpitis patients, Iran. J. Basic Med. Sci. 20 (2017) 209–215.

[33] S.S. Jing Sun, Qun Wang, Kezhou Yu, Rong Wang, Continuous hemodia ltration therapy reduces damage of multi-organs by ameliorating of HMGB1/TLR4/ NFκB in a dog sepsis model, Int, J. Clin. Exp. Pathol. 8 (2015) 1555–1564.