Vasoactive intestinal peptide induces CD14$^+$HLA-DR$^{-/low}$ myeloid-derived suppressor cells in gastric cancer

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Abstract. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of cells, which have been revealed to inhibit T-cell responses in tumor-bearing mice. In addition, a number of immune suppressive mechanisms have linked MDSCs and the development of human cancer. However, the role of MDSCs in human gastric cancer tissue remains to be elucidated as specific markers are lacking. Therefore, the aim of the present study was to investigate the frequency and immune suppressive function of MDSCs denoted in the present study as cluster of differentiation 14 (CD14)$^+$human leukocyte antigen (HLA)-DR$^{-/low}$ in gastric cancer patients. In the present study, MDSCs were directly isolated and characterized from the tumor and adjacent normal tissue of gastric cancer patients. Functional analysis of the CD14$^+$HLA-DR$^{-/low}$ MDSCs co-cultured with allogeneic CD4$^+$ T cells were performed and compared with controls. In addition, the interferon-γ (IFN-γ) and interleukin (IL)-2 production was compared in order to investigate the capacity of vasoactive intestinal peptide (VIP) to induce CD14$^+$HLA-DR$^{-/low}$ MDSC-mediated CD4$^+$ T-cell dysfunction and whether IL-10 secretion is involved in this mechanism. As a result, the quantity of CD14$^+$HLA-DR$^{-/low}$ cells in tumor tissue from gastric cancer patients was significantly higher than that in the adjacent normal tissue. In addition, CD14$^+$HLA-DR$^{-/low}$ MDSCs isolated from tumor tissue were observed to inhibit the CD4$^+$ T-cells' immune responses in comparison with those from the adjacent normal tissue. Furthermore, VIP was able to induce the differentiation of CD14$^+$ mononuclear cells isolated from healthy donor peripheral blood mononuclear cells into activated MDSC cells. Of note, the immunosuppressive effect of VIP-induced CD14$^+$HLA-DR$^{-/low}$ MDSCs on CD4$^+$ T cells was mediated by IL-10 secretion, which was demonstrated in the subsequent decrease of IFN-γ and IL-2 production. In conclusion, CD14$^+$HLA-DR$^{-/low}$ cells were significantly increased in gastric cancer tissue and were shown to have a critical role in CD4$^+$T-cell immunosuppression. In addition, VIP as a novel cytokine may induce the differentiation of CD14+ mononuclear cells towards CD14$^+$HLA-DR$^{-/low}$ MDSCs. An improved understanding of phenotypic heterogeneity and the mechanism of generation of MDSCs in gastric cancer patients is important in the design of effective immunotherapeutic strategies.

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Abbreviations: MDSC, myeloid-derived suppressor cell; VIP, vasoactive intestinal peptide; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; PGE, prostaglandin E; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; PBMC, peripheral blood mononuclear cell; IFN, interferon; PAC, pituitary adenylate cyclase; iNOS, inducible nitric oxide synthase; ARG, arginase; ROS, reactive oxygen species; JAK, janus kinase; STAT, signal transducers and activators of transcription

Key words: gastric cancer, vasoactive intestinal peptide, myeloid-derived suppressor cells

Introduction

Accumulating numbers of studies have demonstrated that myeloid-derived suppressor cells (MDSCs), which were initially documented as 'immature myeloid cells (IMCs)' or 'myeloid suppressor cells', have an important role in immune dysfunction, including promotion of angiogenesis, tumor cell invasion and metastases in human cancer (1,2). In addition, MDSC invasion has been revealed to be critical in patients with cancer, including head and neck cancer, non-small cell lung cancer and renal cancer (3-5). Recent studies have reported that T cell-associated non-response generated from MDSC accumulation was the protagonist of immune tolerance (6). Furthermore, extensive studies have demonstrated that these suppressor cells were of myeloid origin and the heterogeneous cell population comprises myeloid progenitor

cells and IMCs (7). At present, MDSCs are characterized by their particular phenotype and functional ability to suppress T-cell activation.

By contrast to that observed in murine species, MDSCs in humans are inadequately characterized due to a lack of uniform markers. In gastrointestinal cancer patients, MDSCs exhibit diverse phenotypic combinations comprising cluster of differentiation (CD)11b$^+$CD14$^-$ or CD11b$^+$CD33$^+$ human leukocyte antigen (HLA)-DR$^{-/low}$ and can be further discriminated by their expression of CD15 (8). In addition, identification of CD14$^+$HLA-DR$^{-/low}$ MDSCs in melanoma and hepatocellular carcinoma patients provided evidence that different human tumors are likely to induce different populations of MDSCs (9). Two major classes of MDSCs have been identified: Granulocytic and monocytic MDSCs; these two suppressive MDSC subsets may inhibit T-cell responses through alternative mechanisms. The latter, which is characterized by their additional expression of CD14 and reduced expression of CD15, involves a complex network of immune suppression of CD4$^+$ T cells (10).

Multiple pathways in human cancer have been demonstrated to be associated with the recruitment, expansion and activation of MDSCs (7). Considering the multitude of immune modulatory factors produced by tumors, it is likely that different subsets of MDSCs may be generated in the tumor microenvironment, dependent upon the unique profile of factors secreted by the tumor (11,12). Accordingly, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, IL-1β, prostaglandin E2 (PGE2), tumor necrosis factor (TNF)-α and vascular endothelial growth factor (VEGF) were considered to have a potent capacity in the generation of suppressive CD33$^+$ MDSCs (7,11). In addition, a number of laboratories have investigated the critical role of vasoactive intestinal polypeptide (VIP) in the tumorigenesis process (13-15). VIP and its receptors are present in numerous tissues and have an important role in the regulation of endocrine and exocrine secretions, modulation of glucose homeostasis, neuroprotection, memory, gut function, modulation of the immune system and circadian function (16,17). Recent evidence has demonstrated the involvement of VIP in proliferation, adhesion, migration, invasion and cyclooxygenase-2 expression in prostate cancer cells (13). An additional study was performed to identify the contribution of VIP to the expression of VEGF and human epidermal growth factor receptor 2 as well as transactivation of epidermal growth factor receptor in human breast and prostate cancer (14,15), suggesting that VIP has a potent effect in the mechanism of immune tolerance. Furthermore, treatment with VIP resulted in a substantial reduction in the number of CD4$^+$ T cells producing effector cytokines IL-2, IL-4, interferon (IFN)-γ and TNF-α, whereas VIP increased the number of IL-10- and TGF-β-producing CD4$^+$ T cells (18).

Therefore, the frequency and suppressive function of CD14$^+$HLA-DR$^{-/low}$ MDSCs in gastric cancer tissue was evaluated in the present study. In addition, it was investigated whether the presence of VIP induces the differentiation of healthy donor CD14$^+$ peripheral blood mononuclear cells (PBMCs) towards immune suppressive MDSCs with the capacity to inhibit tumor-specific T-cell activation. Understanding their mechanism of action is important for developing effective immunotherapy strategies.

Materials and methods

Patients with cancer and healthy donors. Tumor and adjacent normal tissue (at least 5 cm distant from the tumor margin) were obtained from 19 patients with gastric cancer treated at the Department of Gastrointestinal Surgery of Union Hospital in Wuhan, China. All patients were pathologically diagnosed and none of the patients had received anti-cancer therapy prior to surgical resection. For *in vitro* suppression and induction experiments, blood samples of healthy donors were collected from Wuhan Blood Center (Wuhan, China). Written and oral consent was obtained prior to blood and tumor sampling. The Institutional Review Board of Tongji Medical College of Huazhong University of Science and Technology approved the study protocol.

Cell isolation. A single-cell suspension was isolated from a freshly resected tumor sample and adjacent normal tissue using a mechanical procedure and enzymatic digestion. Subsequently, mononuclear cells were isolated from resulting cells by Ficoll density gradient centrifugation (Ficoll-Paque Plus; eBioscience, San Diego, CA, USA) as described previously (9). PBMCs were isolated from freshly obtained healthy blood samples by Ficoll density gradient centrifugation as described above. The cell viability was assessed using trypan blue dye exclusion. For isolation of CD14$^+$ cells and CD4$^+$ T cells, mononuclear cells were purified using the Human CD14 Positive Selection kit and the Human CD4$^+$ T Cell Enrichment kit (EasySep™ cat. nos. 18058 and 19052 respectively; STEMCELL, Vancouver, BC, Canada) according to the manufacturer's instructions. The purity of the sorted cells was assessed using flow cytometry (BD FACSCanto II; Becton-Dickinson, Franklin Lakes, NJ, USA) and sorted cell populations that were >95% pure target cells were selected for the experiments.

Antibodies and flow cytometric analysis. To determine the frequency and phenotype of CD14$^+$HLA-DR$^{-/low}$ cells in tumor samples and adjacent normal tissue, mouse monoclonal anti-human CD3 (cat. no. 17-0037; 0.6 μg/ml), mouse monoclonal anti-human CD14 (cat. no. 45-0149; 5.0 μg/ml) and mouse monoclonal anti-human HLA-DR (cat. no. 9012-9952; 2.5 μg/ml) antibodies were used to incubate the tissue for 40 min at 4˚C. All monoclonal antibodies used in the study were purchased from eBioscience. Staining was performed on mononuclear cells isolated from tumor-infiltrating tissue or adjacent normal tissue. Fluorescence-activated cell sorting data were acquired using a flow cytometer (BD FACSCanto II; Becton-Dickinson) and were analyzed using BD FACSDiva version 6.1.3 (Becton-Dickinson). Results were expressed as the percentage of CD14$^+$HLA-DR$^{-/low}$ cells.

Suppression assay. To demonstrate the inhibitory capacity of the CD14$^+$HLA-DR$^{-/low}$ MDSCs in gastric cancer tissue, CD14$^+$ mononuclear cells were isolated from tumor-infiltrating tissue and co-cultured with healthy donor CD4$^+$ T cells in the presence of mouse anti-human CD3 (cat. no. 555336;

2.5 μg/ml; BD Biosciences) and mouse anti-human CD28 (cat. no. 555725; 1.2 μg/ml; BD Biosciences) stimulation at 37˚C and 5% CO_2 for five days. The co-culture system comprised complete medium consisting of RPMI 1640 supplemented with fetal bovine serum (10%), L-glutamine (200 mM), sodium pyruvate (1%), nonessential amino acids (1%), penicillin-streptomycin (1%) and 2-mercaptoethanol (1%). As controls, $CD14^+$ mononuclear cells were isolated from adjacent normal tissue and co-cultured with healthy donor $CD4^+$ T cells under identical conditions. After five days, all the cells were harvested in the 24-well flat-bottom plates. For intracellular cytokine staining, cells were exposed for 4 h to phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma-Aldrich, St. Louis, MO, USA), ionomycin (500 ng/ml; Sigma-Aldrich), Golgi-Stop (1 μl/1.5 ml; BD Biosciences) and Golgi-Plug (1 μl/ml; BD Biosciences) as described in a previous study by our group (19). For immunosuppression analysis, the fluorochrome-conjugated antibodies mouse monoclonal anti-human CD4 (cat. no. 47-0047; 0.6 μg/ml; eBioscience), mouse monoclonal anti-human IFN-γ (cat. no. 502506; 5.0 μg/ml; BioLegend, San Diego, CA, USA) and rat monoclonal anti-human IL-2 (cat. no. 500342; 2.5 μg/ml; BioLegend, San Diego, CA, USA) were used for staining at 4˚C for 40 min. Flow cytometric analyses were performed as described above.

Induction assay of VIP. The healthy $CD14^+$ mononuclear cells and $CD4^+$ T cells were isolated from healthy donor PBMCs using Ficoll density gradient centrifugation and magnetic-activated cell sorting as described (9,20). The induction capacity of VIP was analyzed in a co-culture assay with purified healthy $CD14^+$ mononuclear cells ($1.5x10^5$) in the presence of different concentration of pretreated VIP (Null, 10^{-7} M and 10^{-6} M; EMD Chemicals, Inc., Gibbstown, NJ, USA) for three days, as VIP has been observed to induce the emergence of T-regulatory cells (Treg) with suppressive activity on effector T cells and 10^{-7} M VIP was suggested in the induction assay in their studies (18). Subsequently, pretreated $CD14^+$ mononuclear cells ($1x10^5$) were harvested for co-culture with allogeneic $CD4^+$ T cells ($3x10^5$) in the presence of anti-CD3/anti-CD28 stimulation. After five days, all cells were harvested and incubated in the presence of PMA, ionomycin, Golgi-Plug and Golgi-Stop at 37˚C and 5% CO_2 for 4 h. For immunosuppressive analysis, fluorochrome-conjugated antibodies, including anti-CD4, anti-IFN-γ and anti-IL-2 were used for staining and flow cytometric analysis were performed as described above.

Determination of IL-10 expression using ELISA. For determination of IL-10 responses of $CD4^+$ T cells affected by VIP-induced $CD14^+$ PBMCs, co-culture supernatants from the VIP induction assay were removed and assessed using ELISA (Human IL-10 ELISA kit, Invitrogen Life Technologies, Carlsbad, CA, USA). To further confirm the hypothesis that IL-10 secretion is involved in $CD4^+$ T-cell immune tolerance, rat anti-human IL-10 (cat. no. 16-7108; 15.0 μg/ml; eBioscience) was added into the VIP-pretreated $CD14^+$ PBMCs and $CD4^+$ T-cell co-culture system in an induction assay *in vitro*. After five days of co-culture, anti-CD4, anti-IFN-γ and anti-IL-2 were stained and flow cytometric analysis was performed for detecting whether anti-IL-10 can reverse the immunosuppressive capacity of VIP-induced $CD14^+HLA\text{-}DR^{-/low}$ MDSCs.

Statistical analysis. Statistical analysis was performed using SPSS version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). Comparisons were made using Student's t-test for ELISA and the Wilcoxon test for the other data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Frequency of $CD14^+HLA\text{-}DR^{-/low}$ cells is increased in gastric cancer tissue. In general, phenotypic features are considered the hallmark in identifying MDSCs; however, there is no uniform marker for human MDSCs. Previously, CD14 and HLA-DR markers have been partially used to characterize MDSCs in human cancer and identification of more specific surface markers would facilitate the understanding of the origin and functional activities of MDSCs (9,21). $CD33^+$ and $CD11b^+$ expression was suggested as possible surface markers of monocytic MDSCs (22). However, it is noteworthy that PBMCs were widely used for phenotypic analysis of human cancer (9,21-22). Additional experiments towards MDSCs in the tumor microenvironment in humans are required. To investigate the features of immune-associated cells in the tumor microenvironment, the frequency of $CD14^+HLA\text{-}DR^{-/low}$ cells in mononuclear cells isolated from tumor-infiltrating tissue were analyzed in gastric cancer patients. As controls, adjacent normal tissue was assigned as the non-tumor infiltrating group. In the present study, paired tissue was acquired from the same gastric cancer patient to avoid errors caused by individual differences. Representative dot plots of tumor-infiltrating tissue and adjacent normal tissue are shown in Fig. 1A. As shown in Fig. 1B, there was a significant increase in the frequency of $CD14^+$ HLA-DR-/low cells in tumor-infiltrating tissue as compared with that in adjacent normal tissue (13.2±8.0% versus 8.3±2.8%, n=19). As is generally consistent with the results in human cancer PBMCs (9), it was additionally suggested that although the $CD14^+HLA\text{-}DR^{-/low}$ cells observed in tumor and non-tumor infiltrating tissue exhibited common surface markers, the phenotypic heterogeneity and functional difference in immune suppression should be considered (23).

$CD14^+$ $HLA\text{-}DR^{-/low}$ MDSCs are potent suppressors of $CD4^+$ T cells in regard to IFN-γ and IL-2 production. As the suppressive capacity of T-cell immune responses is considered critical in MDCS identification, $CD14^+HLA\text{-}DR^{-/low}$ cells in the present study were analyzed using a suppression assay. In hepatocellular carcinoma patients, $CD14^+HLA\text{-}DR^{-/low}$ cells apparently suppressed T-cell responses in IFN-γ production, whereas $CD14^+HLA\text{-}DR^+$ cells failed to suppress IFN-γ secretion. Therefore, the immunosuppressive function of $CD14^+$ mononuclear cells isolated from tumor PBMCs is predominantly supplied by $CD14^+HLA\text{-}DR^{-/low}$ cell immunosuppression. Subsequently, it was assessed whether $CD14^+$ mononuclear cells from tumor infiltrating tissue of gastric cancer patients are more immunosuppressive to $CD4^+$ T-cell responses, compared with those from non-tumor infiltrating tissue. As a result, $CD14^+$ mononuclear cells from tumor infiltrating tissue suppressed IFN-γ expression of healthy donor $CD4^+$

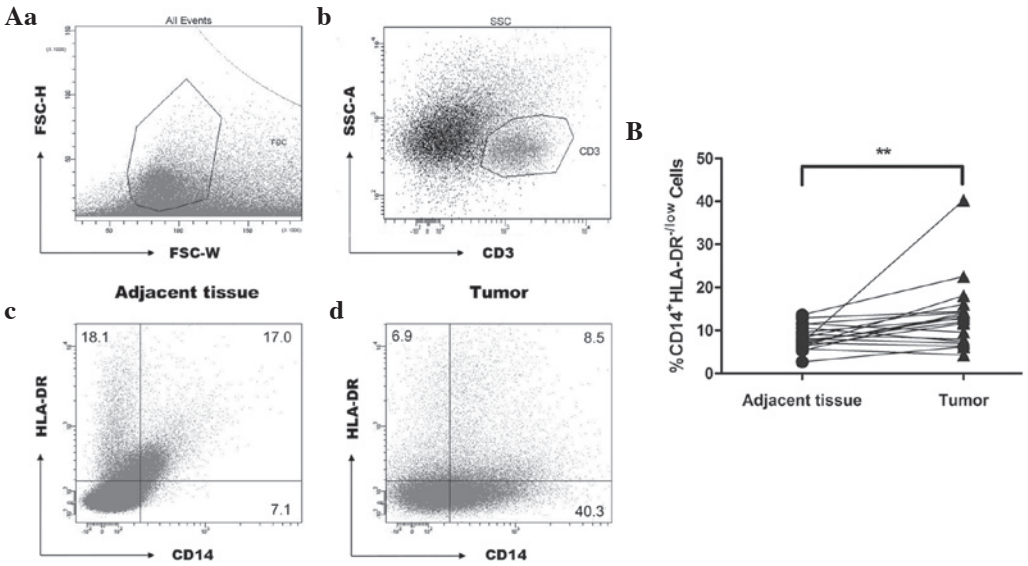

Figure 1. Frequency of $CD14^+HLA\text{-}DR^{-/low}$ myeloid-derived suppressor cells is significantly increased in tumor-infiltrating tissue in gastric cancer patients. Mononuclear cells of gastric cancer patients were stained for CD14 and HLA-DR expression in paired tumor-infiltrating tissue and adjacent normal tissue. CD14 and HLA-DR expression were analyzed by flow cytometry. Values are expressed as the percentage of $CD14^+HLA\text{-}DR^{-/low}$ cells in CD3- cells. Gating strategy (Aa and b) and representative dot plots (Ac and d) of tumor-infiltrating tissue and adjacent normal tissue are shown. (B) Frequency of $CD14^+HLA\text{-}DR^{-/low}$ population in mononuclear cells of tumor-infiltrating tissue and adjacent normal tissue from gastric cancer patients were determined. Values are expressed as the mean ± standard deviation (n=19). $^{**}P<0.01$. CD14, cluster of differentiation 14; HLA, human leukocyte antigen.

T cells, compared with $CD14^+$ mononuclear cells isolated from non-tumor infiltrating tissue (16.2±1.3% versus 8.1±2.3%, n=6; Fig. 2A and B). In addition, as a pleiotropic cytokine with important effects on innate and adaptive immunity, a reduction in IL-2 expression indicated deficient T-cell function and tumor progression. As shown in the tumor microenvironment, $CD14^+$ mononuclear cells suppress IL-2 production of $CD4^+$ T cells, indicating that $CD14^+$ $HLA\text{-}DR^{-/low}$ MDSCs obtained immunosuppressive capacity, whereas $CD14^+$ mononuclear cells from adjacent normal tissue failed to suppress IL-2 production (13.7±2.1% versus 8.2±2.5%, n=6; Fig. 2C and D). The aforementioned findings confirmed that $CD14^+HLA\text{-}DR^{-/low}$ MDSCs caused immune-promoting cytokine downregulation in T-cell immune deficiency in gastric cancer tissue.

VIP is involved in the induction of human $CD14^+HLA\text{-}DR^{-/low}$ MDSCs. Previously, VIP was considered to have potent anti-inflammatory effects and was demonstrated to induce the release of IL-10 and downregulate the number of IFN-γ^+ natural killer (NK) and NKT cells, which subsequently inhibited the cytolytic activity of NK cells (24). One hypothesis is that VIP exerts crucial effects in the pathogenesis of various human tumors, including the initiation, expansion and activation of diverse immune tolerance-associated cells, then trigger the anti-inflammation and tolerance mechanism. To investigate the capacity of VIP in inducing $CD14^+HLA\text{-}DR^{-/low}$ MDSCs and whether the induced MDSCs acquired a suppressive function against $CD4^+$ T cells, freshly sorted healthy $CD14^+$ PBMCs were incubated with VIP at different concentrations. Subsequently, the pretreated $CD14^+$ PBMCs and healthy $CD4^+$ T cells were co-cultured and the suppressive function was measured by IFN-γ and IL-2 downregulation in $CD4^+$ T cells. As a control, null-VIP-pretreated $CD14^+$ PBMCs were used. Compared with the null-VIP pretreatment group, IFN-γ production of $CD4^+$ T cells was inhibited following co-culturing with 10^{-7} or 10^{-6} M VIP-pretreated $CD14^+$ PBMCs (15.1±3.1% versus 9.8±1.4% versus 9.2±1.5%, n=5; Fig. 3A and B). In addition, a similar suppressive effect was detected on the IL-2 production of $CD4^+$ T cells (37.7±7.2% versus 30.4±4.3% versus 29.9±3.7%, n=5; Fig. 3C and D). Accordingly, it was suggested that VIP-pretreated $CD14^+$ PBMCs acquired similar suppressive features to those of $CD14^+$ mononuclear cells isolated from the tumor-infiltrating tissue of gastric cancer tissue. Thus, it was considered that they differenciated into $CD14^+HLA\text{-}DR^{-/low}$ MDSCs, which constituted the suppressive effectors. However, the induction effect was observed to be VIP dose-independent (Fig. 3B and D). The receptor saturation effect may exist in the VIP-mediated induction of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs as VIP receptors, including VIP receptor (VPAC)1, VPAC2 and procaspase-activating compound 1 receptors (18,24). In agreement with this finding, $CD4^+$ T cells incubated in the presence of VIP expressed significantly lower levels of the effector cytokines IL-2, IL-4, IFN-γ and TNF-α (18). Results from these studies suggested the involvement of VIP in immune tolerance through the $CD14^+HLA\text{-}DR^{-/low}$ MDSC induction effect.

Involvement of IL-10 secretion in immune suppression by VIP-induced $CD14^+HLA\text{-}DR^{-/low}$ MDSCs. Several studies have recently proposed the possibility of IL-10 dependent-Treg induction and macrophage inactivation, which may indirectly inhibit tumor cell cytotoxicity mediated by NK cells (25,26). In addition, a more robust type 1 response with increased levels of IFN-γ and decreased levels of IL-10 was observed in a zoledronic acid-treated pancreatic adenocarcinoma murine model due to impaired intratumoral MDSC accumulation and increased recruitment of T cells to the tumor (27). Thus, it was hypothesized that IL-10 secretion was altered in $CD4^+$ T cell response

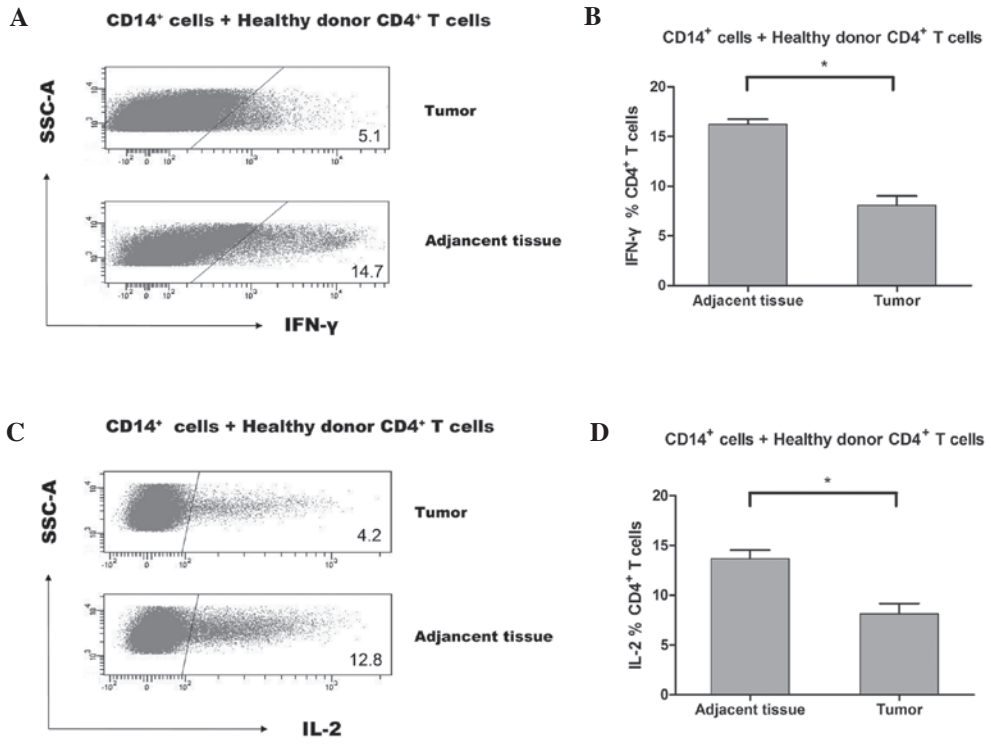

Figure 2. $CD14^+$ mononuclear cells from tumor infiltrating tissue of gastric cancer patients are more immunosuppressive to $CD4^+$ T-cell responses, compared with those from adjacent normal tissue. $CD14^+$ mononuclear cells were sorted using a CD14-positive selection kit and allogenic $CD4^+$ T cells were sorted from peripheral blood mononuclear cells of healthy donors; the two types of cell were co-cultured for five days in the presence of anti-CD3/anti-CD28. Subsequently, the cells were harvested and expression of IFN-γ was determined. (A) Representative dot plot or (B) cumulative results from six independent experiments. In addition, the levels of IL-2 were analyzed and (C) one representative dot plot or (D) cumulative results from six independent experiments are shown. Values are expressed as the mean ± standard deviation (n=6). $^*P<0.05$. CD, cluster of differentiation; IL, interleukin; IFN, interferon.

deficiency caused by VIP-induced $CD14^+HLA\text{-}DR^{-/low}$ MDSCs. As was expected, elevated levels of IL-10 were identified in the supernatants of the co-culture system of VIP-pretreated $CD14^+$ PBMCs and $CD4^+$ T cells (215.7±15.2 pg/ml versus 263.3±29.2 pg/ml or 333.8±81.4 pg/ml; Fig. 4A), compared with those in $CD14^+$ PBMCs without VIP pretreatment. Of note, VIP dose-independence was observed again as no difference was revealed between the 10^{-7} M and 10^{-6} M VIP pretreatment groups (Fig. 4A). It was further hypothesized that the $CD4^+$ T-cell response deficiency may rely on IL-10 secretion when co-cultured with VIP-pretreated $CD14^+$ PBMCs. Therefore, anti-IL-10 was added into the co-culture system and the suppressive progress was reversed and convalescent levels of effector cytokines, IFN-γ and IL-2 were observed. In samples subjected to VIP pretreatment, IFN-γ and IL-2 expression were downregulated (Fig. 4B and D). Statistical data are shown correspondingly in Fig. 4C and E. In anti-IL-10 cases, 10^{-7} M VIP was selected in pretreatment owing to the dose-independent effect in $CD4^+$ T-cell response deficiency between the 10^{-7} M and 10^{-6} M VIP pretreatment groups. These findings indicated that the immunosuppressive effect of VIP-induced $CD14^+$ $HLA\text{-}DR^{-/low}$ MDSCs on $CD4^+$ T cells is IL-10 secretion-dependent.

Discussion

MDSCs have been identified as a potent suppressor of tumor immunity and therefore have potential for cancer immunotherapy. They arise from myeloid progenitor cells that do not terminally differentiate into mature status under the induction by tumor-secreted and host-secreted factors. Different subsets of MDSCs perform different features in morphology, phenotype, gene expression and mechanism of immune tolerance.

In mice, the phenotypic features of these cells were initially defined as $Gr1^+CD11b^+$ and recent studies have unraveled the actual complexity of this population and the existence of granulocytic and monocytic MDSC subsets by distinguishing them into a $CD11b^+Ly6G^+Ly6C^{low}$ and $CD11b^+Ly6G^-Ly6C^{high}$ phenotype (28,29). However, this complexity is more marked in a human setting, where heterogeneous populations of myeloid cells with variable phenotype and immunosuppressive features have been described in different tumors. As a result, numerous MDSC-associated surface markers involved in human tumors were reported in a recent study (10). In humans, monocytic MDSCs are frequently defined as cells expressing the common monocytic marker CD14, but lacking markers of the expression of mature myeloid and lymphoid cells as the major histocompatibility complex class II molecule HLA-DR (9,30). Although a similarity between the CD14 phenotype and morphology exists between monocytic MDSCs and inflammatory monocytes, these cell populations are functionally distinct; monocytic MDSCs are highly immunosuppressive, expressing high levels of inducible nitric oxide synthase (iNOS) and arginase (ARG)1, although these two proteins are not coordinately upregulated in inflammatory monocytes (31). In the analysis of the peripheral blood leukocytes, a previous study observed that there was no difference in the percentage of $CD14^+$ monocytes in non-small cell lung cancer (NSCLC) patients and healthy controls, whereas the percentage and

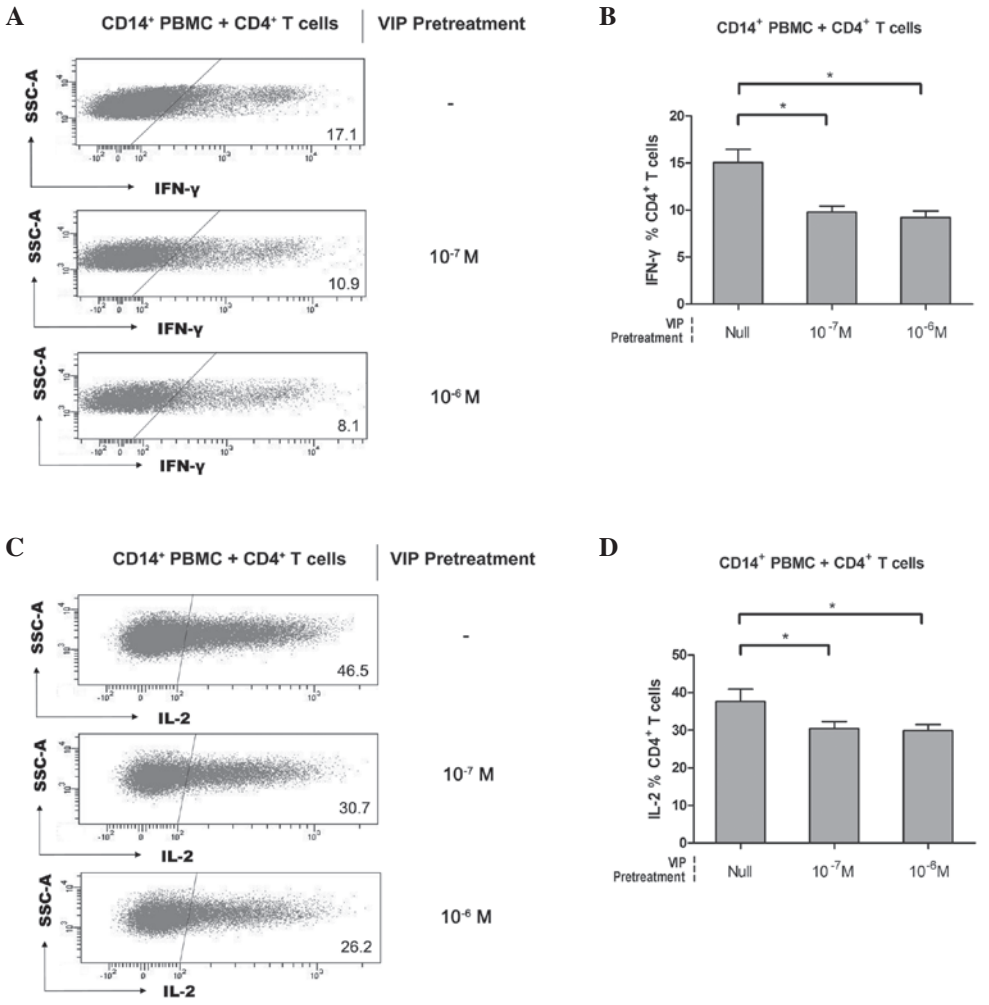

Figure 3. VIP induces CD14+ PBMC conversion into monocytic myeloid-derived suppressor cells and inhibits cytokine-producing potency of healthy donor CD4+ T cells. Freshly sorted CD14+ PBMCs from healthy donors were incubated with VIP at different concentrations (Null, 10^{-7} M or 10^{-6} M). Subsequently, these pretreated CD14+ PBMCs were co-cultured with healthy donor CD4+ T cells. After five days co-culture and stimulation in the presence of anti-CD3/anti-CD28, IFN-γ expression of CD4+ T cells was determined. (A) Representative dot plot or (B) cumulative results from five independent experiments. Following similar VIP pretreatment and co-culture procedures, IL-2 expression of CD4+ T cells was analyzed and (C) one representative dot plot or (D) cumulative results from five independent experiments are shown. Values are expressed as the mean ± standard deviation (n=5). *P<0.05. PBMCs, peripheral blood mononuclear cells; CD, cluster of differentiation; IL, interleukin; IFN, interferon; VIP, vasoactive intestinal peptide.

absolute number of the circulating CD14+HLA-DR$^{-/low}$ subset was significantly increased in NSCLC patients compared with that in healthy controls (4). This further confirmed negative or low expression of HLA-DR as a phenotypic definition of immunosuppressive MDSCs. In a similar way to HLA-DR, the IL-13 receptor α1 chain has also been applied for distinguishing suppressive from non-suppressive myeloid cells, in the same way as Treg was distinguished from activated T effector cells by FoxP3 and CD39 (11,32). In the present study, a novel subset of CD14+HLA-DR$^{-/low}$ MDSCs was described in the tumor microenvironment of gastric cancer patients. CD14 and HLA-DR were previously used as surface molecules of monocytic MDSCs, not only in gastrointestinal tumors, but also in others (33). In another study on the ability of human tumor cell lines to induce MDSCs from healthy donor PBMCs, induced MDSCs were characterized into two distinct subsets: CD33+HLA-DR$^{-/low}$ and CD11b+HLA-DR$^{-/low}$ (22). In addition, MDSCs have also been identified within a CD15+ population in bone marrow and peripheral circulation of pancreatic adenocarcinoma patients (27). On the basis that monocytic MDSCs are discrepantly immunosuppressive compared with granulocytic MDSCs, CD33 is possibly not a discriminatory surface marker for MDSCs, as no difference was observed when MDSCs were further gated for CD33 and CD11b positivity, while CD11b+CD33+ and CD11b+CD33- populations obtained an equal suppressive capacity (8). Recently, several other surface molecules have been used to identify additional subsets of suppressive MDSCs, including CD83 and DC-Sign (markers associated with mature or differentiated cells), with marked expression on CD14+HLA-DR$^{-/low}$ cells in cancer patients (30). Lechner *et al* (22) demonstrated that CD33+ MDSCs are generated when particular cytokines are present in various cancer cell lines. In addition, a particular surface marker was presented in a different proportion in the gated CD14+HLA-DR$^{-/low}$ population; therefore, it was hypothesized that diversity is subsistent between surface markers even if they represent the same population. Therefore, monocytic MDSCs are a population with universal features of phenotypic heterogeneity in gastric cancer.

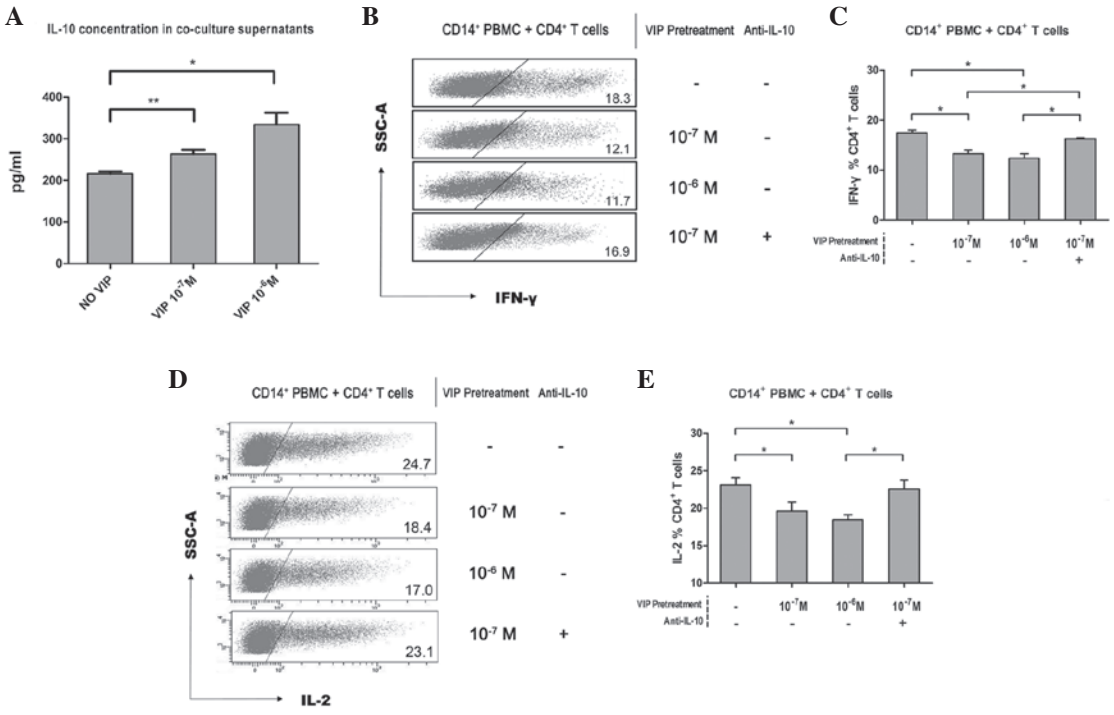

Figure 4. IL-10 secretion is involved in the immunosuppression of VIP-induced CD14+ PBMC to CD4+ T-cell responses. In the VIP induction assay, the co-culture supernatant of CD14+ PBMC and CD4+ T cells was harvested, then its IL-10 concentration was analyzed using ELISA. (A) Cumulative results from eight independent experiments. Furthermore, freshly sorted CD14+ PBMC from healthy donors were pretreated with VIP at different concentrations (Null, 10^{-7} M or 10^{-6} M); these pretreated CD14+ PBMC were then co-cultured with healthy donor CD4+ T cells with/without anti-IL-10 presence. A total of four groups were categorized depending on their VIP pretreatment and anti-IL-10 presence (No VIP + No anti-IL-10, 10^{-7} M VIP + No anti-IL-10, 10^{-6} M VIP + No anti-IL-10, 10^{-7} M VIP + 15 μg/ml anti-IL-10). (B and D) After five days co-culture and stimulation in the presence of anti-CD3/anti-CD28, IFN-γ or IL-2 expression of CD4+ T cells determined and representative dot plot or (C and E) cumulative results from five independent experiments are shown, respectively. Values are expressed as the mean ± standard deviation (n=5). *P<0.05. PBMCs, peripheral blood mononuclear cells; CD, cluster of differentiation; IL, interleukin; IFN, interferon; VIP, vasoactive intestinal peptide.

On the matter of origin, MDSCs are not as simple as a group of migrating myeloid precursor cells, but a cluster of cells that arise from myeloid progenitor cells and do not terminally differentiate into mature status under the induction of tumor-secreted and host-secreted factors under pathological conditions and then obtain an immune suppressive function. Demonstration of the universal nature of solid tumors induced by MDSCs indicated that healthy donor PBMCs can be induced to form immunosuppressive MDSCs via cytokines created whilst bearing a tumor (22). Additionally, CD14+ PBMCs from healthy donors acquired MDSC-like immunosuppressive features following culturing with human glioblastoma cell lines, which tend to promote the apoptosis of autologous T cells (34). From these findings, it was hypothesized that normal human CD14+ PBMCs would adopt an MDSC-like suppressive behavior in response to exposure to tumor-secreted cytokines or host-secreted tumor-induced factors.

The types of factors in pathological conditions involved in the generation of MDSCs, including GM-CSF, IL-6, IL-1β, VEGF, PGE2 and TNFα, were characterized by their capability to transfer the differentiation of myeloid progenitor cells to immune tolerance status (11,12). More recently, a series of studies revealed that VIP can modulate innate and adaptive immunity, demonstrating a predominant anti-inflammatory action against macrophages, promoting a positive T helper(Th)2/Th1 cytokine balance and enhancing the production of Treg (35). In addition, numerous lines of evidence suggested that VIP and its receptors, which are highly expressed in breast tumor cells and lung cancer, have an important role in the pathogenesis of tumors (14,36). Furthermore, natural anti-VIP antibodies, which cause suppression of VIP, may have a protective role against breast and prostate cancer (37). Therefore, it is important to determine the undercover mechanism underlying VIP immunosuppression. In previous studies, IL-2 has been observed to have a critical role in T-cell proliferation, IFN-γ production and cytotoxicity and has been applied in cancer immunotherapy as it can enhance various immune responses, including the generation of antigen-specific T cells, survival rates of memory CD8+ T cells and induction of the cytotoxic T lymphocytes and lymphokine-activated killer cells against tumor cells (38,39). In addition, IFN-γ downregulation is considered critical for T-cell dysfunction. Alongside the results of a previous study by our group (20), the present study supports a crucial role for VIP as a promoter of immune tolerance. It suppresses secretion of IFN-γ and IL-2 as well as immune responses through inducing normal CD14+ PBMCs to monocytic MDSCs. Although the mechanisms involved in the suppressive effect of monocytic MDSCs on the immune system remain controversial, direct or indirect mechanisms possibly involved in the suppressive impact of MDSC on immunity, including the positive correlation between MDSC and Treg levels and inhibiting effect of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs on autologous NK-cell cytotoxicity and cytokine secretion (18,24). In previous studies, the ability of MDSCs in promoting $CD4^+CD25^+FoxP3^+$ Treg cells *in vivo* was

described, which contributes to the indirect immunosuppression of T-cell responses by MDSCs (26,33). Furthermore, the involvement of VIP in immune tolerance through the induction of Treg was confirmed (18,40). Of note, this Treg-inducing progression involved TGFβ-dependent and -independent pathways (34,41). Dendritic cells (DCs) are a type of effective immune cell and it has been confirmed that anti-inflammatory IL-10 interferes with DC maturation (42). Upregulation of IL-10 and poor stimulation of allogeneic T cells was observed in the differentiation from DC to tolerogenic DC (43). In addition, it was suggested that $CD14^+HLA\text{-}DR^{-/low}$ monocytes were capable of inhibiting T-cell proliferation and DC maturation (44). Thus, MDSCs may inhibit T-cell responses and DC activity by upregulating the production of IL-10.

To expose the details of IL-10 involvement in MDSC activation, Sinha *et al* (24) demonstrated that MDSCs impaired immunity by promoting a type 2 response, in which $CD4^+$ T-cell and $CD8^+$ T-cell responses were skewed through interacting with macrophages to increase IL-10 expression (24). In addition, macrophage-produced IL-12 was shown to promote NK activity and MDSCs may indirectly mediate a reduction in the production of IL-12 by macrophages, relying on their IL-10 production; thus, increased IL-10 production may also indirectly inhibit tumor cell cytotoxicity mediated by NK cells, which is a type of important effector cell in tumor immunity (45). As a result, MDSCs directly suppress adaptive and innate anti-tumor immunity and facilitate tumor growth through their cross-talk with macrophages. The present study demonstrated that VIP immunosuppression on $CD4^+$ T cells is mediated by $CD14^+HLA\text{-}DR^{-/low}$ MDSC induction, which is consistent with increased secretion of IL-10. According to initial studies, IL-10 is a pleiotropic cytokine with important immunoregulatory functions and it possesses potent anti-inflammatory properties (46); it represses the expression of inflammatory cytokines, including TNF-α, IL-6 and IL-1β in macrophages. IL-10 and its receptor may be involved in tyrosine phosphorylation of signal transducer and activator of transcription (STAT)3 by the receptor-associated Janus kinase (JAK)1 and tyrosine kinase 2, then subsequently trigger JAK-STAT pathways (46-48). Several different signaling pathways in MDSCs were apparently relevant to transcription factors of the STAT family, mainly comprising STAT1, STAT3 and STAT6. Recent findings indicated that the characteristics of pathological processes may recruit different subpopulations of MDSCs with different mechanisms and targets of suppression. As described in the review by Gabrilovich and Nagaraj (7), the granulocytic subset of MDSCs was found to express high levels of ROS and low levels of NO, whereas the monocytic subset expressed low levels of ROS and high levels of NO, and the two subsets expressed ARG1. In a study by Kusmartsev *et al* (49), MDSCs from $STAT^{-/-}$ mice failed to upregulate the expression of ARG1 and iNOS and therefore did not inhibit T-cell responses, which suggested that STAT1 was the main transcription factor involved in the upregulation of ARG1 and iNOS expression by monocytic MDSCs in the tumor microenvironment. In addition, it has been demonstrated that MDSCs may inhibit T-cell function through Th2 cytokine IL-13 as another pathway independent of ARG1, iNOS or ROS (50). All of the above indicates a complex interaction network between MDSCs and T-cell responses. Drawing conclusions from the present study requires the consideration whether VIP is involved in the pathological processes above. Thus far, the present study provided the presumption that IL-10 is involved in the generation of MDSCs from $CD14^+HLA\text{-}DR^{-/low}$ cells treated with VIP, which may be accompanied by STAT family activation; however, further investigations are required to examine the signaling pathway associated with MDSC generation in human gastric cancer.

In conclusion, an increased frequency of immunosuppressive MDSCs in the tumor microenvironment of gastric cancer was reported in the present study. MDSCs characterized by a $CD14^+HLA\text{-}DR^{-/low}$ phenotype in these patients may suppress tumor-specific $CD4^+$ T-cell responses, confirming a crucial immunosuppressive pathway of MDSCs developed in gastric cancer patients. In the present study, it was further proposed that VIP, a novel cytokine, which can induce the differentiation to MDSCs which have a immunosuppressive function via the upregulation of IL-10 production, leading to suppression of the T-cell response. In addition, the results of the present study suggested the differentiation of gastric cancer cells into monocytic MDSCs. Although the present study does not rule out every conceivable pathway, it provided a clear association between VIP, monocytic MDSC and T-cell dysfunction. Further analysis of the mechanism of VIP-induced MDSC immune suppression may assist in the search for novel targets for cancer immunotherapy.

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