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Increased Numbers of Tumor-lysing Monocytes in Cancer Patients

Claudia Papewalis ¹, Benedikt Jacobs ¹, Anna M. Baran ¹, Margret Ehlers ¹, Nikolas H. Stoecklein ²
 Holger S. Willenberg ¹, Sven Schinner ¹, Martin Anlauf ³, Andreas Raffel ², Kenko Cupisti ², Roland
 Fenk ⁴, Werner A. Scherbaum ¹, and Matthias Schott ¹

¹ Endocrine Cancer Center, Department of Endocrinology, Diabetes and Rheumatology, University
 Hospital Duesseldorf, Duesseldorf, Germany

² Department of General-, Visceral- and Paediatric Surgery, University Hospital Duesseldorf,
 Duesseldorf, Germany

³ Department of Pathology, University Hospital Duesseldorf, Duesseldorf, Germany

⁴ Department of Haematology, Oncology and Clinical Immunology, University Hospital Duesseldorf,
 Duesseldorf, Germany

Short title: CD14+/CD56+ monocytes in endocrine cancer patients

Correspondence to:

Claudia Papewalis, PhD
 Matthias Schott, MD, PhD
 Department of Endocrinology, Diabetes and Rheumatology
 University Hospital Duesseldorf
 Moorenstr. 5
 40225 Duesseldorf, Germany
 Tel: +49 211 8104038, FAX: +49 211 8117860
 email: claudia.papewalis@uni-duesseldorf.de
 matthias.schott@med.uni-duesseldorf.de

keywords: monocytes, natural killer cells, CD56, cytotoxicity, interferon- α

abbreviations: DC, dendritic cell; IFN-DC, interferon- α generated DC; PBMC, Peripheral blood
 monocyte; PTC, papillary thyroid carcinoma

disclosure: all authors have nothing to disclose

Abstract

Lymphatic infiltration is a well known phenomenon in different tumors including endocrine malignancies. However, little is known about the role of antigen-presenting cells and T cell activation in this context. The aim of our study was to investigate the quantity and function of CD14+/CD56+ monocytes in tumor patients including endocrine malignancies. First, these cells were characterized in peripheral blood of endocrine and non-endocrine cancer patients as well as in tumor tissue samples. Cancer patients had in mean 3.7 times more CD14+/CD56+ monocytes in the peripheral blood compared to healthy controls ($p = <0.0001$), while the highest frequencies were seen in patients with heavy tumor load. Importantly, these cells additionally expressed several NK cell markers. A proof of CD14+/CD56+ infiltrations into papillary thyroid carcinoma was shown by immunohistochemical analyses. Functional analyses revealed an apoptosis inducing capacity *in vitro* after IFN- α re-stimulation. Our data indicate the importance of tumor-lysing monocytes in antitumor immunity.

1 Introduction

2 Natural occurring anti-tumor immune response is a well known phenomenon in different non-endocrine
 3 (Dalerba et al., 2003; Jorkov et al., 2003; Lang et al., 1977; Sander et al., 1996; Waldner et al., 2006)
 4 and endocrine malignancies. In endocrine and endocrine-related malignancies this is mainly known for
 5 papillary thyroid carcinoma (PTC) and also for ovarian cancer (Adams et al., 2009; Stumpf et al., 2009;
 6 Zhang et al., 2003). In PTC the immune response appears to be important in preventing metastasis
 7 and recurrence of thyroid cancer (Davies et al., 1992; Gupta et al., 2001; Juhasz et al., 1989; Kamma
 8 et al., 1988; Misaki et al., 1992; Okayasu et al., 1995). This is based on several observations. First,
 9 patients suffering from autoimmune thyroiditis in parallel to PTC generally have improved disease-free
 10 survival (Loh et al., 1999; Ozaki et al., 1996). Second, a lymphocytic infiltration of adult PTC is
 11 associated with lower disease-stage at diagnosis and a reduced recurrence risk (Matsubayashi et al.,
 12 1995). Third, several studies have shown that children and adolescents with PTC have a more
 13 favorable prognosis than adults (McClellan et al., 1996; Welch Dinauer et al., 1998). Thereby, it has
 14 been demonstrated that PTC of children and adolescents contain more lymphocytes than PTC of
 15 adults, and that PTC patients exhibiting the highest proliferating capacity of lymphocytes have the best
 16 prognosis (Gupta et al., 2001). All together, these observations emphasize the importance of
 17 lymphocytic infiltration and proliferation in preventing thyroid cancer recurrence.

18 In contrast to these data, almost nothing is known about the initial step of antitumor immunity in regard
 19 tumor cell recognition, tumor cell lysis and tumor antigen-presentation to T cells. One study described
 20 an increased expression of co-stimulatory molecules B7-1 and B7-2 in PTC of children and
 21 adolescents (Shah et al., 2002). This is important as B7-2 co-activates T cells by binding its CTLA-4
 22 receptor. In line with these data, an increased frequency of antigen-presenting immature dendritic cells
 23 (DC) has also been described in differentiated, poorly differentiated, and undifferentiated / anaplastic
 24 thyroid carcinomas, respectively (Ugolini et al., 2007).

25 Most recently we identified a new sub-population of DCs with properties of natural killer (NK) cells
 26 (Papewalis et al., 2008). These cells are mainly characterized by the expression of the NK cell marker
 27 CD56, a direct lysis capacity towards tumor cells via tumor necrosis factor-related apoptosis-inducing
 28 ligand (TRAIL) and a strong activation of T cells. These cells may, therefore, represent a link between
 29 innate immunity and adaptive anti-tumor immunity. These DCs can be generated *in vitro* from
 30 monocytes by exposure to interferon- α [IFN- α ; (Papewalis et al., 2008)] and are, therefore, termed as
 31 CD56+ IFN-DCs. The aim of the present study was to look for the presence of precursors of these
 32 cells in the peripheral blood of tumor patients as well as in tumor lesions and to characterize their
 33 potential role in the initiation of naturally occurring anti-tumor immune response in endocrine
 34 malignancies.

Material and Methods

Reagents

All products used for cell culture were purchased from Invitrogen Life Technologies GmbH (Karlsruhe, Germany). For complete medium RPMI 1640 was supplemented with GlutaMAX™, 10% fetal calf serum (FCS), 100 U/ l penicillin, and 100 µg/ l streptomycin. Recombinant endotoxin- and BSA-free human IL-2 was purchased from R&D Systems (Wiesbaden, Germany). Fluorochrome labeled antibodies were purchased by BD Bioscience (Heidelberg, Germany) except anti-CD56/57 and anti-BDCA1/2 (Miltenyi Biotec, Bergisch Gladbach, Germany), and NKG2A (R&D Systems). For immunohistochemical analyses reagents were purchased from Dako (Hamburg, Germany) if not otherwise indicated.

Phenotypic analysis by flow cytometry analysis

Peripheral blood monocytes (PBMCs) from patients or healthy volunteers were collected and isolated by Vacutainer® Cell Preparation Tubes (BD Bioscience). Phenotyping was performed after blocking FC receptors by labeling entire cells with anti-CD14 PerCP (clone MΦP9) and anti-CD56 APC (AF12-7H3), and staining several DC and NK markers using appropriate antibodies which were FITC or PE labeled. DC surface markers were characterized by CD80 (L307.4), CD83 (HB15e), CD86 (IT2.2), CD40 (5C3), CD11c (S-HCL-3), CD123 (9F5), CD209 (DCN46), CD1a (SK9), CD1c (BDCA 1; clone AD5-8E7), CD303 (BDCA 2; AC144), and HLA-DR (G46-6). Additionally for examination of NK cell surface markers, antibodies were used towards CD16 (NKP15), CD94 (HP-3D9), CD161 (DX12), as well as anti-CD159a (NKG2A; clone 131411), CD314 (NKG2D; 1D11), CD337 (NKp30; P30-15), CD336 (NKp44; P44-8.1), and CD335 (NKp46; 29A1.4). All staining was performed in parallel to appropriate isotype controls using a FACS-Comp device supplemented with the Cell Quest PRO software (BD Biosciences). A minimum of 10.000 events per region of interest (R1 = monocyte cell region) was measured.

Blood samples

Altogether, 95 blood samples of cancer patients (n = 55) and healthy controls (n = 40) have been included into the study. All blood samples were collected in the morning hours. Cancer patients were in mean 60.1 ± 14.5 years old (26 male patients) and suffered from endocrine malignancies including metastasized dedifferentiated thyroid cancer, gastrointestinal neuroendocrine cancer and adrenal cancer. Non-endocrine cancer patients suffered from colon cancer, multiple myeloma, acute myeloid leukemia and B cell lymphoma. Healthy controls were in mean 33.2 ± 12.3 years old (20 male persons). Detailed patients characteristics are given in Table 1. The study has been approved by the Local Ethical Committee of the Heinrich-Heine-University Duesseldorf.

Immunohistochemical analyses of tumor specims

Sections of tumor biopsies from PTC patients (n = 5; two were delivered with an additional infiltrated lymph node) were analyzed by immunohistochemical staining. Serial sections of 6 µm were prepared, dried, fixed by acetone for 10 min at -20°C, and incubated with 5% porcine serum for 1h. Over night, slides were incubated with a polyclonal rabbit antibody towards CD14 and a monoclonal mouse

antibody towards CD56 (clone MOC-1; both from Abcam Cambridge, UK) and serial sections were incubated with rabbit anti-CD3 and anti-CD8 antibodies. All antibodies were diluted 1:50 in antibody diluent with background reducing components; incubation was performed at 4°C. The following day, secondary labeling was performed by using Alexa[®] fluoro-dye antibodies (molecular probes[®], Invitrogen) differently fluorescence labeled (anti-goat 488; anti-mouse 594; anti-rabbit 488 or 594, each at a concentration of 1:500). Finally, DNA was counterstained by 4',6-Diamidino-2-phenylindol containing ProLong Gold[®] antifade reagent (Invitrogen). Signals were visualized by a Kenence microscope BZ8000 affiliated to a digital camera. The multipoint time-lapse software BZ-H1MT enables a subsequent digital reconstruction of the pictures implicating a stereoscopic view.

Enrichment of monocytes for functional analyses

Preparation of highly purified monocytes was performed from blood of patients with endocrine and neuroendocrine tumors (n = 3) which were identified before for high amounts of CD14+/CD56+ monocytes. For the enrichment the magnetic micro-bead separation technology was used (Miltyen Biotec, Bergisch Gladbach, Germany). PBMC were isolated as described above and incubated with FC-blocking reagent for 10 min within MACS buffer. Thereafter, T cells, granulocytes, NK cells, and B cells were depleted by biotin-labeled antibodies towards CD3 (clone UCHT1), CD66b (G10F5), CD57 (TB03), CD161 (DX12), and CD19 (1D3) for 15 min. Afterwards, cells were washed twice and incubated with FC-blocking reagent again for 10 min to prevent unspecific attachment of antibody-labeled NK cells from monocytes which would otherwise result in high contamination of NK cells after cell sort. Finally anti-biotin micro-beads were added for 15 min, washed again, and pasted through a LS column (both Miltenyi Biotec). Negatively selected cells were prepared for an additional FACS sorting by labeling the cells with anti-CD14 FITC, a cocktail of PE-labeled antibodies towards CD3, CD19, CD57, CD66b, CD161, and anti-CD56 APC for 10 min together with FC-blocking reagent and propidium iodide to exclude death cells. Labeled cells were suspended in PBS/ EDTA-Medium (2 mM EDTA, 2% FKS) at a concentration of 3×10^7 cells per ml to monocytes with a purity of 100% monocytes and a viability of >98% by a BD FACSAria[™] II.

Stimulation of monocytes for cytotoxicity assays

Monocytes (1×10^6 per ml in 24-well-plates) were stimulated in complete medium for 18 hours with 1.000 U/ml GM-CSF and 10.000 U/ml IFN- α (Roferon-A; Roche, Basel, Switzerland). Thereafter, cells were washed and used for apoptosis detection or cytotoxicity assays.

Cell lines

Jurkat (ACC 282) and PC3 (ACC 465) cell lines were purchased from the German Resource Centre for Biological Material (DMSZ) in Braunschweig, Germany.

Apoptosis detection

Jurkat cells and monocytes were harvested and washed twice with PBS. A co-incubation was performed with monocytes at a ratio of 1:1 in 200 μ l complete medium in a 96-well V-shape plate for 18h. Afterwards, cells were harvested and washed twice in an Annexin binding buffer (BD Bioscience). Then, cells were resuspended in 100 μ l buffer and incubated with 5 μ l anti-Annexin FITC (BD

Bioscience) and 5 μ l propidium iodide (Sigma) for 15 min. For analyzing, 400 μ l binding buffer was added and the percentage of Annexin V-expressing tumor cells was analyzed by flow cytometry. Thereby, tumor cells were gated and analyzed by their greater FSC/ SSC-values compared to monocytes. A positive control for Annexin V staining was implanted by incubation cell samples with camptothecin (6 μ M for 4 h at 37°C).

Cytotoxicity assay

K562 and PC3 cells were harvested, washed with PBS and subsequently incubated with chromium-51 (3.7 kBq in 100 μ l containing 1×10^6 cells) for 1 hour. Thereafter, cells were washed twice in PBS and 5×10^3 tumor cells were incubated with monocytes at different ratios as effectors in 200 μ l complete medium in a 96-well V-shape plate. For positive control cells were lysed with Tween 20 which was than defined as total lysis (100%); for negative control cells were cultured in normal medium without lysing reagents. After 8 hours 100 μ l supernatant was taken and chromium release was analyzed by a Wizard Automatic Gamma Counter (Wallac, Turku, Finland). Data were expressed as the means $\pm$ SD of triplicate wells. The percentage of cytotoxicity was calculated as: cytotoxicity = [(experimental group cpm - spontaneous cpm) / (total cpm - spontaneous cpm)] $\times$ 100.

Statistical analysis

The results were analyzed for statistical significance by unpaired t-test with Welch's correction while no equal variances were assumed. Analyses were performed using the Prism computer software (GraphPad Software).

Results

Phenotypic analysis of peripheral CD14+/CD56+ monocytes by flow cytometric analysis

Investigation of PBMC of cancer patients (n = 55) and healthy volunteers (n = 40) revealed a significant higher number of CD14/CD56 double positive monocytes in patients suffering from tumors (mean $9.5\% \pm 1.6\%$) compared to normal controls ($2.6\% \pm 0.27\%$; Figure 1A) resulting in a 3.7 times higher frequency of these cells in cancer patients compared to healthy volunteers.

A more detailed analysis of the cancer patients subgroups revealed nearly identical number in endocrine cancer patients ($13.8\% \pm 4.9\%$) compared to non-endocrine cancer patients with solid tumors ($9.2\% \pm 2.3\%$; n.s.). Importantly, however, there was a wide range of double positive cells in endocrine malignancies from 0.2% up to 53.5%. Largely increased numbers ($> 5\%$ of all monocytes) were seen in 8 out of 13 (61.5%) patients suffering from endocrine malignancies. Within this group there were patients with metastatic poorly differentiated thyroid cancers (n = 2) as well as patients with intestinal neuroendocrine cancers (n = 3) and patients with adrenal cancer (n = 2). Six of those (irrespective of their origin) even had CD14+/CD56+ cell numbers of $>10\%$ of all monocytes. The highest frequencies were seen in the two patients suffering from poorly differentiated thyroid cancer (53.5% and 33.2% CD14+/CD56+ cells) as well as in one patient with a metastasized neuroendocrine cancer of intestinal origin (22.2% ; Figure 1B). In addition, we also found a correlation between the tumor load and the presence of CD14+/CD56+ monocytes. All patients with high cell numbers suffered from extended metastatic disease with liver and bone metastases and in 5 out of 7 cases also with lung metastases. In contrast, increased numbers ($> 5\%$) of CD14+/CD56+ monocytes were seen only

in 4 out of 8 (50%) patients with limited tumor spread (defined as morphological detectable metastases in one additional organ at maximum). These data indicate that proliferation and activation of CD14+/CD56+ monocytes depends on the tumor load and to a less extent on the tumor origin at least in the three endocrine cancer types enrolled in this study.

Age-related differences between cancer patients and healthy controls could be excluded by re-analysis of older healthy volunteers (age ≥ 50 years, $n = 7$) compared to the youngest volunteers (age ≤ 25 years, $n = 8$). Here, similar amounts of double positive cells were found, as $3.0 \pm 1.5\%$ double positive cells were seen in cancer patients compared to $2.5 \pm 1.2\%$ in young healthy volunteers ($p = \text{n.s.}$). In addition, we compared the cell numbers of age-matched healthy controls ($n=20$; mean age 33.2 years) compared to young cancer patients ($n= 6$; mean age 33.3 years); again very similar data were found as controls revealed 2.6% CD14+/CD56+ cells compared to 8.8% in cancer patients ($p= 0.0168$). Based on that, an age-dependent increase of CD14+/CD56+ monocytes can be excluded.

In order to investigate the role of these cells in solid tumors compared to non-solid malignancies, we also included patients with hematological disorders. Here, a similar pattern was seen with frequencies of CD14+/CD56+ monocytes of in mean $8.0\% \pm 1.8\%$ ($p = 0.0024$). In particular, patients suffering from plasmacytoma and acute leukemia ($n = 25$) revealed a more than 3-fold higher amount of CD14+/CD56+ monocytes ($7.9\% \pm 2.3\%$) compared to patients with lymphoma ($n = 6$; $1.9\% \pm 0.6\%$; n.s.).

In addition, we quantified other surface markers of the CD14+/CD56+ monocyte population characteristic for highly potent DCs. Co-stimulatory molecules such as CD80, CD1a, CD40, and CD83 as well as specific markers of plasmacytoid DCs (CD123, CD303) or myeloid DCs (CD209, CD1c) could almost not be detected ($<5\%$) whereas CD86 and HLA-DR were detected on nearly all cells ($>92\%$; data not shown), which is in accordance with the marker constellation of peripheral monocytes. Additionally, the presence of other NK cell markers was investigated. Here, nearly the same results were seen for endocrine cancer patients and for non-endocrine tumor patients as well as healthy volunteers. In cancer patients, in mean $15.9\% \pm 2.1\%$ of CD14+/CD56+ cells were also positive for CD16 in contrast to healthy donors where $21.2\% \pm 2.9$ of these cells were also positive for CD16 (n.s. ; Figure 2B). Some minor differences were seen for the cytotoxicity triggering KIR receptor 3 also known as NKp30 (CD337). In cancer patients in mean $56.8\% \pm 4.6\%$ of all CD14+/CD56+ positive cells were also positive for NKp30 (CD337) versus $39.3\% \pm 5.2$ cells in healthy volunteers ($p = 0.015$). Both markers are classical surface receptors of NK cells (Balsamo et al., 2009; Brandt et al., 2009; Della et al., 2009; Zarife et al., 2009). In contrast, other NK cell markers such as CD314 (NKG2D) and CD94 could not be detected on these cells.

Immunohistochemical analyses of tumor infiltrates

Since lymphocytic infiltration is a well known phenomenon for PTC, tissue sections of PTC patients were used for immunohistochemical staining. All tumor samples showed a strong infiltration of CD3+ and CD8+ T cells. Importantly, we also found CD14+/CD56+ monocytes which were in close vicinity to tumor cells in two tumor specims, one of them within the tumor infiltrating lymph node. One representative confocal microscopy is shown in Figure 3. These data support the assumption of a central role of CD14+/CD56+ monocytes in tumor cell recognition including the induction of peripheral immune tolerance by CD14+/CD56+ monocytes.

Apoptosis detection

In order to investigate the functional capacity of CD14⁺/CD56⁺ double positive monocytes we performed Annexin V testing on the basis of FACS analyses with Jurkat cells as specific targets (van Engeland et al., 1998). As previously described, monocytes may reveal a direct cytolytic activity (Fanger et al., 1999; Griffith et al., 1999; Kirsch et al., 1994; te Velde et al., 1992). Here, monocytes induced apoptosis of in mean $5.0\% \pm 0.3\%$ compared to normal controls ($2.5\% \pm 0.5\%$; $p = 0.0153$). Additional co-culturing with IFN- α resulted in a large increase of CD14⁺/CD56⁺ double positive cell numbers. Co-incubation of these IFN-DCs with Jurkat cells revealed an apoptosis induction of in mean $19.8\% \pm 4.7\%$ (Figure 4A; $p = 0.0065$). Figure 4B depicts a representative FACS analysis showing a strong apoptosis induction of Jurkat cells especially after co-incubation with IFN- α stimulated monocytes (right panel).

Cytolytic activity of CD14⁺/CD56⁺ monocytes

Cytotoxicity assays were performed on the basis of chromium release detection. For this purpose PC3 cells were used as targets because of their TRAIL and Fas ligand sensitivity (Zisman et al., 2001). Here, we could show that co-culturing of tumor cells with IFN-DCs resulted in a direct dose-dependent cytotoxicity of up to $21\% \pm 3.3\%$ (Figure 4C). In accordance, cytotoxicity assays with K562 tumor cells which are only TRAIL sensitive (Park et al., 2009), showed a lysis of up to $17.6\% \pm 1.1\%$ (effector to tumor cell ratio: 80 to 1).

Co-stimulatory molecules and T cell activation

Recently, we already reported on an increased T cell stimulatory capacity of CD56⁺ IFN-DCs (Papewalis et al., 2008). Since co-stimulatory molecules on antigen-presenting cells are important for T cell stimulation, we quantified the expression of CD80 and CD86 molecules. Here, we found a 3.8-fold increase of fluorescence intensity for the surface marker CD80 (from 113.8 ± 21.5 to 431.5 ± 42.7) and a 5.1-fold increase for CD86 (from 201.1 ± 30.2 to 1043 ± 132.9 ; Figure 4D). The upregulation of co-stimulatory molecules may partially explain the increased T cell stimulatory capacity CD56⁺ APCs as previously shown (Papewalis et al., 2008).

Discussion

Tumor-infiltrating lymphocytes are associated with less extensive disease stage at diagnosis and improved disease-free survival in non-endocrine (Adams et al., 2009; Dalerba et al., 2003; Jorkov et al., 2003; Lang et al., 1977; Sander et al., 1996; Stumpf et al., 2009; Waldner et al., 2006; Zhang et al., 2003) and endocrine malignancies e.g. in adult patients with papillary thyroid carcinoma (PTC) (Matsubayashi et al., 1995; Ozaki et al., 1996; Ugolini et al., 2007). Gupta et al. studied 63 cases of PTC in children and young adults and reported that the long-term outcome was more favorable when the lymphocytic infiltration was present both within the tumor and in the surrounding tissue (Gupta et al., 2001). Interestingly, although disease-free survival did not correlate with the numbers of lymphocytes per high power field, survival rate was significantly improved in the case of tumors showing high numbers of Ki-67+ lymphocytes. It was concluded that proliferation of tumor-associated lymphocytes correlates with improved disease-free survival in at least the investigated subset of young patients. More recently, it was found that a high proportion of PTCs arising in children and young adults contains infiltrating CD4+, CD8+, or CD19+ lymphocytes and that the three cell types may be contemporarily present. This suggests that the anti-PTC response is complex and involves more than one class of lymphoid cells (Modi et al., 2003).

Based on the aforementioned results and our previous data regarding the role of DCs in anti-tumor immunity (Papewalis et al., 2008), we were now interested in the role of tumor-lysing and antigen-presenting monocytes in tumor patients e.g. in endocrine cancer patients. Here, we focused on CD14+/CD56+ monocytes as potential precursors of CD56+ DCs (Papewalis et al., 2008). Our present data clearly demonstrate that cancer patients including those with endocrine malignancies reveal much higher numbers of CD14+/CD56+ monocytes compared to healthy controls. Importantly, we also demonstrate that these cells infiltrated into tumor lesions and that these cells show a direct cytolytic activity towards tumor cells upon activation with IFN- α *in vitro*. In addition, we show that IFN- α generated CD56+ DC also up-regulate co-stimulatory molecules necessary for T cell activation. Taken together, these cells may represent a link between the innate and the adaptive immunity as they are able to directly lyse tumor cells and phagocyte antigens to present them to other effector cells. Therefore, we may postulate that these cells are involved in the initiation and maintenance of T cell infiltration into (endocrine) tumors. In contrast, however, we also found a correlation between the magnitude of tumor spread and the amount of double positive monocytes. Therefore, we can neither exclude that CD14/CD56+ monocytes are down-regulated by the tumor environment (Kusmartsev et al., 2006; Nefedova et al., 2005) nor that these cells may directly suppress specific immune responses as one mechanism of peripheral immune tolerance (Zitvogel et al., 2010).

Another important question in this context is the role of cytokines. Two major players are IFN- γ and IFN- α . Experiments in mouse tumor models have shown that IFN- α plays an important role in the activation of a long lasting antitumor response (Belardelli et al., 2002). This might be attributed to its capability to induce the differentiation of the Th1 subsets, the generation of CTL, and in the promotion of *in vivo* T cell proliferation and survival (Kolumam et al., 2005; Marrack et al., 1999; Sun et al., 1998; Trinchieri et al., 1978) as well as the activation of NK cells (Pfeffer et al., 1998). Moreover, we have shown that IFN- α could lead to the maturation and functional activation of monocyte derived DCs which are highly CD56 positive (Papewalis et al., 2008). Therefore, a strong stimulus such as the presence of

1 IFN- α may convert immunosuppressive CD14+/CD56+ monocytes into active players of a cytotoxic
2 immune response again.

3 Within our study, monocytes with strong expression of CD56 were also positive for other NK cell
4 markers including CD16 and CD337. In the past, it has already been demonstrated that monocytes,
5 also positive for CD16, are involved in inflammation and sepsis (Belge et al., 2002; Blumenstein et al.,
6 1997; Fingerle et al., 1993; Scherberich et al., 1999). Here, these cells have a high potential in antigen
7 presentation which is mainly due to the strong expression of HLA-DR molecules (Belge et al., 2002;
8 Ziegler-Heitbrock 2007). In contrast, the role of CD337 on CD56+ monocytes is highly unclear. CD337,
9 also known as NKp30 or NCR3, is a natural killer receptor of NK cells. In contrast to all other NK killing
10 receptors, CD337 is mainly involved in the crosstalk between NK cells and DC resulting in both, an
11 activation or an inhibition of DCs by CD337+ NK cells (Ferlazzo et al., 2002; Gerosa et al., 2002;
12 Piccioli et al., 2002). This effect, mainly depends on the cell to cell ratio (Piccioli et al., 2002). Whether
13 the CD337+/CD56+ monocytes may also have a regulatory function is yet unknown. The direct
14 correlation between an increased cell number and metastatic spread may support the
15 immunoregulatory / immunosuppressive function of these cells. On the other hand, co-incubation of
16 these cells with IFN- α clearly showed a direct cytotoxic function towards tumor cells indicating the
17 potency of these cells to induce a cytotoxicity-directed immune response. Importantly, CD14/CD56-
18 expressing DCs described here are different from those initially described by our group (Papewalis et
19 al., 2008) since those cells were negative for all other NK cell markers (except CD56) including CD337
20 and CD16. These differences can easily be explained by the precursor cells used for analyses. While
21 we now used the purified CD14+/CD56+ monocyte subset for our analyses, we formerly focused on
22 the complete CD14+ monocyte population which has than be used for incubation with IFN- α .

23 We also investigated the mechanisms of tumor cell lysis by CD14+/CD56+ monocytes. For this
24 purpose, two different tumor cell lines were included: the PC3 cell line was used because of its TRAIL
25 and Fas ligand sensitivity (Zisman et al., 2001) and K562 tumor cells were used because of their pure
26 TRAIL sensitivity (Park et al., 2009). Co-incubation of IFN- α activated CD56+ expressing monocytes
27 with PC3 cells resulted in a similar lysis capacity of tumor cells compared to co-incubation with K562
28 tumor cells. These data indicated that both mechanisms, at least however TRAIL, are involved in the
29 cytolysis of tumor cells by CD14+/CD56+ monocytes.

30 In summary, we report for the first time on CD14+/CD56+ monocytes in tumor patients. These cells
31 reveal a direct cytolytic function towards tumor cells in vitro. These cells may represent precursors of
32 CD56+ IFN-DCs which are able to efficiently activate T cells. We also report on an increased
33 frequency of these cells in tumor patients in general including endocrine cancer patients and could
34 demonstrate that these cells infiltrate into tumor lesion. At moment, we can not exclude that these cells
35 may also reveal an immunoregulatory / immunosuppressive effect because of a direct correlation with
36 tumor spread. The induction of a cytotoxic immune response after co-culturing with IFN- α may,
37 however, support the potency of these cells to convert into direct cytolytic players in anti-cancer
38 immunity.

Figure 1: Frequency of CD56+ monocytes in the peripheral blood of tumor patients

(A-B) Phenotypic analyses of freshly isolated peripheral blood mononuclear cells. (A) CD56+ monocytes were monitored in the peripheral blood of 40 volunteers (healthy controls) and 55 patients with solid tumors (n=18) or lymphatic malignancies (n=37) shown as “all tumors”. Detailed analyses of non-endocrine solid tumors (n=5) and endocrine tumors (n=13) did not show major differences. (B) Representative FACS analysis: After FC-receptor blocking, cells were stained with monoclonal antibodies towards CD14 and CD56. For detailed analysis, monocytes were gated (R1; upper left panel) and analyzed for double positive cells. The amount of these cells was quantified. Representative FACS analyses for one healthy volunteer (upper right panel) and for two patients suffering from different solid tumors (lower panels) are shown.

Figure 2: Detailed FACS analyses of CD56+ monocytes for dendritic cell and NK cell surface markers

(A) Monocytes phenotypically gated in R1 (upper left panel) were gated further for CD14 and CD56 double positive cells (R2; upper middle panel), and were analyzed for dendritic cell (DC) and NK cell surface markers. Corresponding isotype controls were performed in all tests (upper right panel). Two representative FACS analyses for CD337 expression in two patients are shown in the lower panels. (B) Summarized data of analyses of NK markers on CD56+ monocytes: while the amount of some markers (CD314 and CD94) did not significantly differ between healthy volunteers (open dots) and tumor patients (closed dots), NK markers (CD337 and CD16) were considerably different.

Figure 3: Confocal microscopy of lymphocytic infiltrations in papillary thyroid carcinomas

(A-B) Serial sections (6 μ m) of a patient. Magnifications are indicated: (A) 400x and (B) 800x. Serial sections were stained for CD3+ and CD8+ T cells (upper middle and right panels). Here, we found infiltrating CD3+ T cells which were stained green ($\lambda = 488$) as well as CD8+ T cells which were marked red ($\lambda = 594$). Moreover, serial sections were also stained for CD14 ($\lambda = 488$; green) and for CD56 ($\lambda = 594$; red) (lower left and middle panels). Confocal microscopy revealed increased numbers of CD14+/CD56+ cells (green and red) which emerge in an orange phenotype (lower right panels; arrows). These cells are in close vicinity to tumor cells of the PTC. Isotype control and secondary antibody incubation showed no fluorescence neither for Alexa-488 nor for Alexa-594 using the same exposure time (lower panels).

Figure 4: Functional analysis of CD56+ monocytes

(A-B) Annexin analysis of Jurkat cells which were co-incubated with freshly isolated or IFN- α - stimulated monocytes (n = 3). (A) Jurkat cells were incubated for 18h either with freshly isolated or IFN- α stimulated monocytes at a ratio of 1:1. Apoptosis of Jurkat cells was induced in $5.0\% \pm 0.3\%$ of all cells after co-incubation with monocytes and in $19.8\% \pm 4.7\%$ of all cells after co-incubation with IFN- α stimulated monocytes. Controls were performed by incubation of Jurkat in complete medium alone. (B) Representative flowcytometric analysis shows Jurkat cells without treatment (left panel), a positive control by co-incubation of Jurkat cells with camptothecin (6 μ M; middle panel), and a strong apoptosis induction of Jurkat cells after co-incubation with IFN- α stimulated monocytes (right panel). (C) In parallel, PC3 tumor cells were used for incubation with freshly isolated monocytes and IFN- α

1 stimulated monocytes (n = 3). IFN- α stimulation induced a strong, cell number-dependent cytolytic
2 activity (chromium release) of monocytes up to 20.9% $\pm$ 3.3%. **(D)** The increase of lysis activity by co-
3 incubation with IFN- α (1000 U/ml for 18h) goes along with a large increase of co-stimulatory molecules
4 CD80 and CD86 which are necessary for T cell induction.

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Table 1:
Cancer Patients and healthy volunteers enrolled in the study

	Total numbers of patients (n)	Male / female patients (n / n)	mean age (years)	CD14+/CD56+ monocytes (% of all monocytes $\pm$ SD)
healthy volunteers	40	20 / 20	33,2	2.6% $\pm$ 0.27%
cancer patients (all)	55	26 / 29	60,1	9.5% $\pm$ 1.6%
solide tumors	18	4 / 14		
<u>endocrine malignancies</u>	13	2 / 11	66,5	13.8% $\pm$ 4.9%
poorly differentiated thyroid carcinoma	2	0 / 2		
neuroendocrine cancer	4	1 / 3		
adrenal carcinoma	2	0 / 2		
papillary thyroid carcinoma	5	1 / 4		
<u>non-endocrine tumors</u>	5	2 / 3	65,6	9.2% $\pm$ 2.3%
colon carcinoma	2	0 / 2		
Liposarkoma	1	1 / 0		
squamous cell carcinoma	1	1 / 0		
primary tumor unknown	1	0 / 1		
hematological disorders	37	21 / 16	59,4	8.0% $\pm$ 1.8%
multiple myeloma (plasmocytoma)	18	9 / 9		
myelodysplastic syndrome	4	3 / 1		
acute leukemia	6	5 / 1		
Hodgins and Non-Hodgkins lymphomas	7	2 / 5		
chronic myeloproliferative diseases	2	2 / 0		

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Figure 1:

Figure 2:**A** **B**

Figure 3

Figure 3 continued

Figure 4:**A** **B** **C** **D**