Chapter 4

Tumor-infiltrating CD14 positive myeloid cells and CD8 positive T cells prolong survival in patients with cervical carcinoma

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Brief description: This study on the different types of myeloid cells, their clinical impact and their co-operation with T cells offers a profound insight on the role of myeloid cells in the microenvironment and how they can work side by side to control tumors. As such, our data are a major addition to the current discussion about the impact of tumor-infiltrating myeloid cells in human cancers and offer potential new strategies to improve survival.

ABSTRACT

One of the hallmarks of cancer is the influx of myeloid cells. In this study we investigated the constitution of tumor-infiltrating myeloid cells and their relationship to other tumor-infiltrating immune cells, tumor-characteristics and the disease-specific survival of patients with cervical cancer. Triple color immunofluorescence confocal microscopy was used to locate, identify and quantify macrophages (CD14), their maturation status (CD33) and their polarization (CD163) in a cohort of 86 patients with cervical carcinoma. Quantification of the numbers of myeloid cells revealed that a strong intraepithelial infiltration of CD14+ cells, and more specifically the population of CD14+CD33-CD163- matured M1 macrophages, is associated with a large influx of intraepithelial T lymphocytes (p=0.008), improved disease-specific survival (p=0.007) and forms an independent prognostic factor for survival (p=0.033). The intraepithelial CD8+ T-cell and Treg ratio also forms an independent prognostic factor (p=0.010) and combination of these two factors reveals a further increased benefit in survival for patients whose tumor display a dense infiltration with intraepithelial matured M1 macrophages and a high CD8 T-cell/Treg ratio, indicating that both populations of immune cells simultaneously improve survival. Subsequently we made a heatmap including all known immune parameters for these patients, whereby we were able to identify different immune signatures in cervical cancer. These results indicate that reinforcement and activation of the intratumoral M1 macrophages may form an attractive immunotherapeutic option in cervical cancer.

INTRODUCTION

Cervical cancer (CxCa) is caused by high risk human papilloma virus (HPV) (1). Studies on HPV-specific T-cell response in patients with premalignant disease suggest that spontaneous regression occurs when circulating HPV early antigen-specific CD4+ and CD8+ T-cells are present and when the lesions are infiltrated with effector T-cells that outnumber regulatory T-cells. Moreover, the presence of circulating HPV-specific CD4+ T-cells is associated with T-cell infiltration in the lesion and favorable clinical outcome in high-grade squamous intraepithelial lesion (HSIL) after treatment (2;3). The development of CxCa is associated with a weak systemic and local immune response to HPV, reflected by low numbers of tumor-infiltrating T-cells comprising CD8+ cytotoxic T-cells, CD4+ T-helper cells and regulatory T-cells (4-6). The T-cells present often lack cytotoxicity (7) and/or express co-inhibitory molecules such as programmed cell death protein 1 (PD-1), CD94 and NKG2a (8;9). Tumors also down regulate Human Leukocyte Antigen (HLA) class I and MHC class I chain-related molecule A (MICA) and up regulate HLA-E and PD-L1 to further restrain the CD8+ T-cell response (2;8-10). The presence of circulating HPV-specific T-cells associates with better survival and high numbers of T-cells correlate with the absence of metastases or a relapse (2;6;11;12). Importantly, the ratio between tumor-infiltrating CD8+ and Foxp3+ T-cells was found to be the first immune-associated independent prognostic factor in CxCa (2).

Tumors mediate systemic and local effects, altering the accumulation and differentiation of myeloid cells and redirecting their function to sustain tumor outgrowth. Three groups of terminally differentiated myeloid cells are essential for innate and adaptive immunity – macrophages, dendritic cells (DC) and granulocytes – and monocytes are the major precursor of the first two populations in humans. Macrophages are a heterogeneous population of tissue-resident monocytes, which display different functions depending on the microenvironment. Roughly two types are recognized; the classically activated type 1 macrophages (M1) which are tumoricidal and produce IL-12 and the alternative activated type 2 macrophages (M2) which sabotage immunity by producing IL-10, prostaglandin E2 (PgE2), TGFβ and CCL22 (13-15). Extensive literature demonstrates that high numbers of tumor associated macrophages (TAMs) facilitate tumor growth, disease progression and poor prognosis (reviewed in (16)). The development from premalignant cervical lesions towards carcinoma is associated with high numbers of infiltrating CD68+ macrophages (17;18). However, in CxCa TAM were never associated with clinical parameters (12;19;20) or clinical outcome. It is conceivable that differences in macrophage subsets exist between patients, as some CxCa produce PgE2 and IL-6 (21;22), shown to be important for M2 macrophages differentiation (23). Furthermore, there are vast differences between patients concerning density and type of tumor-infiltrating T-cells (2;6), which may also effect the type of macrophages present (24;25).

This study uses a unique cohort of CxCa patients, for which many immune parameters are known, to investigate the presence of infiltrating myeloid cells and their relationship to other tumor-infiltrating immune cells, tumor characteristics and patient survival. We used fluorescence confocal microscopy to quantify macrophages (CD14), their maturation status (CD33) and their polarization (M2; CD163). CD14 is a specific monocyte/macrophage marker, although it can also be found on subsets of dendritic cells (26). CD33 is expressed on non-terminally differentiated myeloid cells (27) and CD163 is linked to macrophage anti-inflammatory functions (26;28;29). We demonstrate that the density of tumor-epithelium infiltrating CD14+CD163- cells is an independent prognostic factor for prolonged disease-specific survival. Furthermore, unsupervised clustering of patients based on 40 known immune markers suggested that a tumor microenvironment that allows the accumulation of high numbers of CD14+CD33-CD163- myeloid cells forms a prerequisite for tumor-infiltrating CD8+ T-cells to exert their antitumor effect.

MATERIALS AND METHODS

Patient Material

Formalin-fixed, paraffin-embedded tissue blocks from 86 CxCa patients undergoing a radical hysterectomy type III with pelvic lymphadenectomy (1985-2000) were retrieved from the Pathology Department (Leiden University Medical Center, Leiden, the Netherlands). Patients had not received radio- or chemotherapy before surgery. A trained pathologist reviewed all H&E-slides. All material was used according to Dutch Federation of Medical Research Associations guidelines.

Immunohistochemistry

Characterization of tumor-infiltrating myeloid cells (TIM) was carried out with triple immunofluorescent staining and confocal microscopy. Anti-CD33 (1:50, mouse-IgG2b, clone PWS44, LeicaMicrosystems, Rijswijk, the Netherlands), anti-CD14 (1:100, mouse-IgG2a, clone 7, Abcam, Cambridge, UK) and anti-CD163 (1:400, mouse-IgG1, Clone 10D6, LeicaMicrosystems) mixture was applied after EDTA (pH 9) pretreatment. CD68 (Mouse-IgG2a, Clone 514H12, Serotec, Düsseldorf, Germany)/CD163 double-staining was also performed. The following fluorescently-labeled antibodies were used: goat-anti-mouse IgG2b-AlexaFluor546, goat-anti-mouse IgG2a-AlexaFluor488 and goat-anti-mouse IgG1-AlexaFluor647 (MolecularProbes, Bleiswijk, the Netherlands). Images were captured with a confocal laser-scanning-microscope (Zeiss LSM 510, Germany) in a multitrack setting. Per slide, five randomly selected images were captured. Negative control slides, omitting the primary antibody, were included. Tumorcell-nests and stroma were measured using the

Zeiss LSMImageExaminer and myeloid subsets were manually counted and presented as number/mm^2.

In vitro differentiation assay of M2 macrophages

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors (29). CD14+ monocytes were isolated using MACS cell-separation (Miltenyi Biotec). Monocytes were cultured with 25 ng/ml M-CSF (R&D systems, United Kingdom) to induce M2 macrophages (23). On day 0 and 8, one sample was taken for FACS analysis and another was paraffin-embedded and stained with CD14 AF700 (clone M5E2, BD Biosciences, the Netherlands), CD33 PEcy7 (clone P67.6, BD Biosciences, the Netherlands) and CD163 APC (clone 215927, R&D systems, United Kingdom) .

Statistical Analysis

Patients were divided into groups based on the median of the infiltrating cells. Cumulative disease-specific survival rate was calculated by the Kaplan-Meier method and analyzed by the log-rank test (Statistical Package for the Social Sciences software package 17 (SPSS)). A Cox regression analysis was used for the univariate and multivariate survival analyses of lymph node status, tumor size, tumor infiltration depth, vasoinvasion and parametria involvement as well as the different types of tumor-infiltrating immune cells. A p-value of <0.05 was considered statistically significant. The two-sided χ^2 and the Fisher's exact tests were used to associate TIMs, clinical variables, tumor-infiltrating lymphocytes (TILs) and expression of other tumor ligands. The Bonferroni correction was applied for multiple analyses. The Pearson's correlation was used within the myeloid cells group.

For the creation of a heatmap, immune cell counts were divided into quartiles, except the CD3+CD8+CD57+ cells which were displayed in two groups due to low positive cell number. Tumor ligand expression was divided into two or three groups: positive or negative (HLA-E (9), HLA-G (unpublished), HLA-class II (2;10;30), MICA (2), PD-L1 (8), Serpin A1/A3 (31)) or negative, weak, strong (Chemokine (C-X-C motif) ligand (CXCL)12 , C-X-C chemokine receptor (CXCR)4 (32), Indoleamine2,3-dioxygenase (IDO) (unpublished), CXCR7 (32), HLA-class I (2;10;30) and epidermal growth factor receptor (EGFR) (33)). The function 'heatmap' of the 'stats' package in R (Development Core Team, a language and environment for statistical computing, reference index version 2.14.0. 2005 Foundation for Statistical Computing, Vienna, Austria) was used. Standard settings were used: euclidean distance and complete-linkage clustering (34). Data on the different lymphocitic sub-populations was previously generated (2;6). Tumor expression of the various ligands was measured by the Ruiter-system (35). Changes from the lowest to the highest quartile are reflected by a darker color, white blocks are missing data.

RESULTS

The CxCa microenvironment comprises a large variety of myeloid cells

Patient characteristics are displayed in table 1. To evaluate the infiltration of TIMs within the tumor-epithelium and stroma 86 tumors were analyzed for CD14, CD33 and CD163. Vast differences in the number and type of myeloid cells was found between tumors (Figure 1 and Table 2). In general, the stroma was more densely infiltrated with TIMs. The most common TIMs within the tumor-epithelium were CD14+CD33-CD163-, CD14+CD33+CD163+ and CD14-CD33-CD163+. These were also abundantly found in the stroma (Table 2). To confirm that the CD14+ cells were macrophages a staining was performed with the macrophage specific marker CD68 as well as CD163 in 10 CxCa. About 70% of the CD68+ cells expressed CD163 (M2 macrophages) (Supplementary Figure S1a). Additionally, in vitro M-CSF-mediated differentiation of monocyte to M2 macrophages showed that these cells clearly expressed CD14 and high levels of CD163 (Supplementary Figure S1b). All together, we showed that tumors are infiltrated with variable M2 (CD14+CD163+) and M1 (CD14+CD163-) macrophage numbers. In both our analyses using CD68 or CD14 as a macrophage marker we observed CD163+ cells that were CD68- (Supplementary Figure S1a) or CD14-, suggesting the presence of non-macrophage CD163+ myeloid cells, also observed by others (29).

Table 1. Patient characteristics

	N **(%)**
No. of patients	86
Average age (years)	49.6
Ethnicity	
Caucasian	74 (86)
Suriname	5 (6)
Unknown	7 (8)
FIGO[1] stage	
1b1	37 (43)
1b2	28 (33)
2	21 (24)
HPV type	
16	45 (52)
18	19 (22)
Other	13 (15)
Negative	9 (11)
Histopathology	
Squamous	53 (61)
Adeno (squamous)	33 (38)
Lymph node metastasis	
Negative	60 (70)
Positive	25 (29)
Unknown	1 (1)
Tumor size	
<4 cm	44 (51)
≥4 cm	36 (42)
Unknown	6 (7)
Infiltration depth	
<15 mm	43 (50)
≥15 mm	42 (49)
Unknown	1 (1)
Vasoinvasion	
No	37 (43)
Yes	44 (51)
Unknown	5 (6)
Parametrial involvement	
No	76 (88)
Yes	10 (12)
Mean follow-up time (months)	49.3
5-year survival rate	65 (76)

[1]International Federation of Obstetricians and Gynecologists.

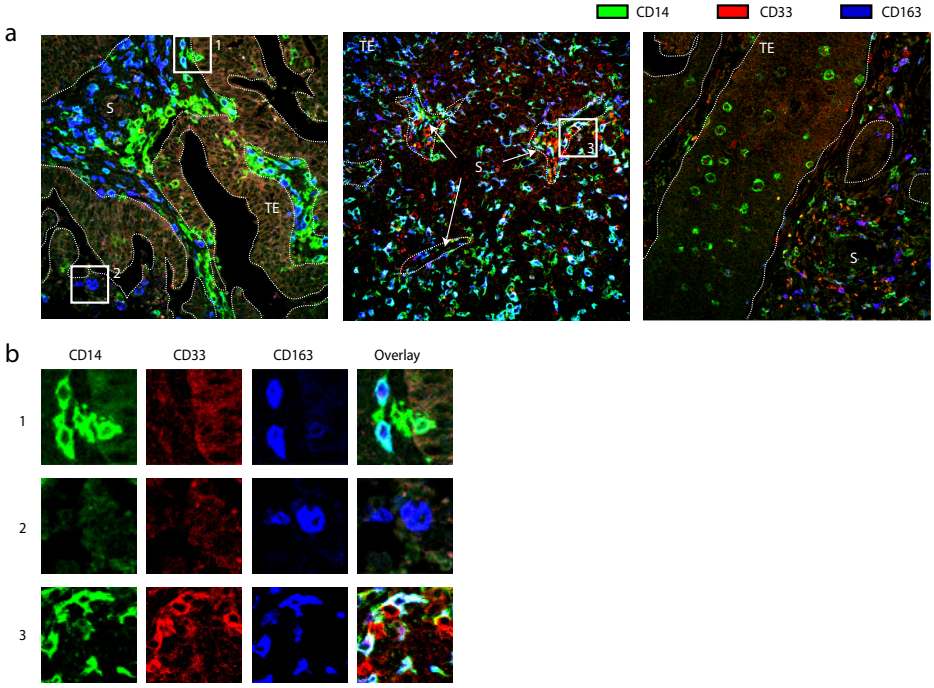

Figure 1. Immunofluoresence staining of cervical carcinoma with antibodies against CD14 (green), CD33 (red) and CD163 (blue). (a) The tumor-epithelium (TE) and tumor stroma (S) are marked. The left panel depicts an example of an adenocarcinoma with dense infiltration of the stroma by CD14+CD33-CD163- (green), CD14+CD33-CD163+ (turquoise) and CD14-CD33-CD163+ (blue) cells. In the middle, a squamouscarcinoma with dense infiltration of the tumor-epithelium by various myeloid cells is shown. In the right panel is an example of CD14+CD33-CD163- cells (green) infiltrating the tumor-epithelium. (b) An example of CD14+CD33-CD163- (green) and CD14+CD33-CD163+ cells (turquoise; number 1) staining; CD14-CD33-CD163+ cells (blue; number 2); various myeloid marker combinations found in close proximity (number 3).

Table 2. Cellular distribution in tumor epithelium and stroma

Cell type[1]	Epithelium, median[2] (interquartile range)	Stroma, median[2] (interquartile range)
CD14+CD33−CD163−	12 (4–28)	40 (20–69)
CD14+CD33+CD163−	4 (0–16)	13 (2–38)
CD14+CD33−CD163+	9 (2–21)	39 (14–84)
CD14+CD33+CD163+	10 (1–49)	59 (14–198)
CD14−CD33+CD163−	3 (0–15)	19 (7–42)
CD14−CD33+CD163+	8 (1–26)	31 (7–72)
CD14−CD33−CD163+	10 (2–26)	52 (15–113)

[1]The identification of different myeloid cell subsets on the basis of the expression of CD14, CD33 and CD163.
[2]Number of cells per mm^2.

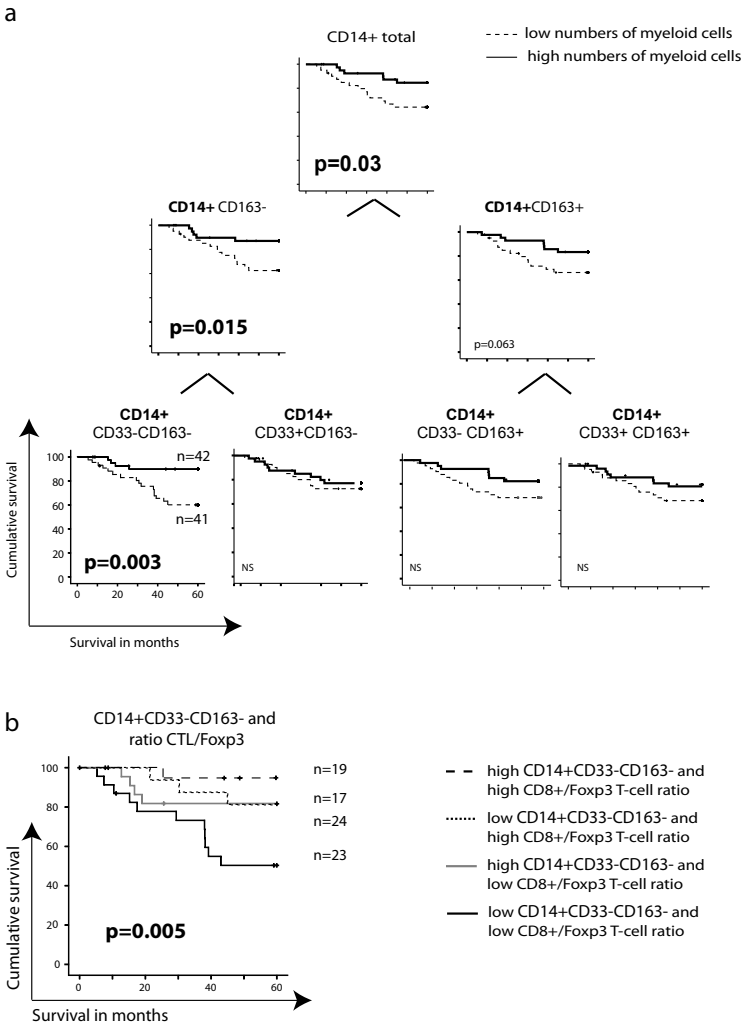

Figure 2. Myeloid cell populations were divided into two groups based on the median number of intraepithelial myeloid cells. (a) Kaplan Meijer curves of the total CD14+ cells showed a significant survival benefit for patients with more than median CD14+ tumor-infiltrating cells. When these cells were divided adding the expression of CD163, the survival benefit was seen in the group of patients with more than median CD14+CD163- infiltrating cells, yet a trend was still seen for the CD14+CD163+ myeloid cells. The main sub-population of the CD14+CD163- cells was CD33- (75%), which was again significantly correlated to a prolonged survival. (b) The ratio of CD8+ and Foxp3+ T-cells infiltrating the tumor-epithelium was previously identified as an independent factor of disease-specific survival in cervical carcinoma patients. The Kaplan Meijer of this ratio combined with the CD14+CD33-CD163- myeloid cells, showed a greatly increased 5-year disease-specific survival for patients with high numbers of both cell types and the poorest disease-specific survival in patients with low numbers of both cell types.

Intraepithelial M1 macrophages are associated with a better disease-specific survival

While on average CxCa is well infiltrated by the three major subsets of myeloid cells, a clear difference exists between individual patients as indicated by the interquartile ranges provided for each cell population (Table 2). To assess the prognostic impact of these myeloid populations, the 5-year Kaplan-Meier estimate and log-rank test were used. Patients were divided into two groups based on the median number of myeloid cell subset per mm^2 in the tumor-epithelium or in stroma.

Firstly the patients were analyzed according to the total myeloid cell population expressing CD14+, CD33+ or CD163+. Patients with tumors displaying high numbers of intraepithelial CD14+ myeloid cells, showed a better 5-year disease-specific survival (p=0.03; Figure 2). No survival benefit was seen based on the number of intraepithelial CD33 or CD163 expressing cells (Supplementary Figure S2). Further division of the CD14+ myeloid cell population based on CD163 expression revealed that patients with high numbers of intraepithelial CD14+CD163- cells (M1 macrophages) displayed a survival benefit (p=0.015). Unexpectedly a trend towards a better survival was seen in the patients with high numbers of intraepithelial M2 macrophages (p=0.063). Pearson's correlation test showed that the numbers of CD14+CD163- and CD14+CD163+ cells were strongly related in both tumor-epithelium (p=0.002) and stroma (p=0.008), suggesting that the observed trend between M2 macrophages and disease-specific survival could be a confounder. The non-macrophage CD14-CD163+ myeloid cells were not correlated to any of the other groups of myeloid cells in epithelium or stroma (0,086 and 0,083 respectively).

Further subdivision based on CD33 expression revealed that especially the number of intraepithelial CD14+CD33-CD163- cells (mature M1 macrophages), which made up 75% of all cells in the CD14+CD163- group (Table 2), was associated with improved disease-specific survival (p=0.003; Figure 2). Notably, the trend between intraepithelial M2 macrophages and disease-specific survival was lost. No associations with survival were found for cells present in stroma.

M1 macrophages and T-cells act together to improve disease-specific survival

Previously we have shown that CxCa can be abundantly infiltrated by CD8+ T-cells, but also by Foxp3+ T-cells (2;6). Therefore, the relationship between the infiltration of the different types of TIMs and the other previously enumerated tumor-infiltrating immune cells in this group of patients was studied. The presence of high numbers of intraepithelial CD14+CD163- cells (predominantly CD14+CD33-CD163- cells), correlated with a high influx of intraepithelial T lymphocytes (p=0.008; Table 3). There was no correlation with any specific lymphocyte subset. In contrast, the intraepithelial population of non-macrophage CD163+ myeloid cells, irrespectively of CD33 expression, was positively correlated to the

Table 3. Correlations to other tumoral and environmental parameters

Intraepithelial myeloid subgroups[1]	CD3+CD8− Foxp3−	CD3+CD8+ Foxp3−	CD3+CD8− Foxp3+	Ratio CD4/ Foxp3	Ratio CTL/ Foxp3	Immune infiltrate
CD14+CD163−	0.382	0.183	0.15	0.32	0.925	**0.008 (0.048)**[2]
CD14+CD33−CD163−	**0.037**	0.24	0.26	0.51	0.82	**0.012**
CD14+CD33+CD163−	0.33	**0.036**	0.69	0.38	0.97	**0.038**
CD14+ CD163+	**0.017**	0.089	0.859	0.65	0.877	**0.03**
CD14+CD33−CD163+	0.27	0.35	0.42	0.32	0.32	0.19
CD14+CD33+CD163+	0.98	0.87	0.59	0.062	0.59	0.27
CD14−CD163+	0.083	0.28	**0.006 (0.036)**[2]	**0.021**	0.062	0.519
CD14−CD33+CD163+	0.49	0.05	0.15	0.059	**0.02**	0.99
CD14−CD33−CD163+	**0.03**	0.12	0.15	0.9	0.87	0.51

	HLA class 1	HLA class 2	MICA	IDO	PDL1
CD14+CD163−	0.887	0.928	0.475	0.586	0.22
CD14+CD33−CD163−	0.43	0.62	0.52	0.27	0.078
CD14+CD33+CD163−	0.22	0.64	0.33	0.52	0.62
CD14+ CD163+	0.194	0.594	0.18	0.726	0.734
CD14+CD33−CD163+	**0.032**	0.81	0.38	0.15	0.96
CD14+CD33+CD163+	**0.027**	0.12	0.47	0.57	0.26
CD14−CD163+	0.78	0.093	0.478	0.44	0.071
CD14−CD33+CD163+	0.2	0.88	0.26	0.73	0.087
CD14−CD33−CD163+	**0.02**	**0.005 (0.025)**[2]	0.81	0.32	0.22

	Negative lymph nodes	Tumor size <4 cm	Infiltration depth <15 mm	No vasoinvasion	No parametrial involvement
CD14+CD163−	0.081	0.632	0.504	0.744	0.165
CD14+CD33−CD163−	**0.009 (0.045)**[2]	0.73	0.58	0.12	**0.043**
CD14+CD33+CD163−	0.7	0.7	0.91	0.48	0.93
CD14+ CD163+	**0.005 (0.03)**[2]	0.685	0.513	0.133	0.143
CD14+CD33−CD163+	0.86	0.27	0.82	0.74	0.54
CD14+CD33+CD163+	**0.007 (0.035)**[2]	0.84	0.38	**0.019**	**0.039**
CD14−CD163+	0.519	0.77	0.513	0.745	0.191
CD14−CD33+CD163+	0.758	0.543	0.827	0.22	0.38
CD14−CD33−CD163+	0.86	0.68	0.82	0.57	0.52

[1]For the assessment of relationships between histopathological parameters and myeloid subpopulations, the latter were divided based on the 50th percentile.
[2]The values in bold indicate the p-values that are considered to be significant, the values between the brackets indicate the *p*-value after Bonferroni correction, which are still considered significant.

number of intraepithelial CD3+CD8-Foxp3+ Tregs (p=0.006; Table 3). No correlations were found between stromal TIMs and intraepithelial immune cells (data not shown).

We have previously shown that it is not a single population of TIL that is associated with survival, but specifically the ratio between the tumor-infiltrating CD8+ and Foxp3+ T-cells (2). We therefore assessed the influence of the intra-epithelial CD14+CD33-CD163- myeloid population and the intra-epithelial CD8+/Foxp3+ T-cell ratio by dividing the patients into 4 groups based on the median number of both cell types.. The best disease-specific survival was seen in the group of patients with high numbers of intraepithelial CD14+CD33-CD163- myeloid cells and a high CD8+/Foxp3 T-cell ratio (n=19, p=0.005; Figure 2b). Conversely, patients with low numbers of these myeloid cells and a low CD8+/Foxp3 T-cell ratio displayed the worst survival (n=23). Interestingly, the groups with only high numbers of CD14+CD33-CD163- myeloid cells or a high CD8+/Foxp3+ T-cell ratio,

did equally well (Figure 2b), suggesting that M1 macrophages and CD8+ T-cells have an additive anti-tumor effect.

We also assessed the association between TIM and expression of HLA-class I/II, MICA (2;30), IDO (36;37) and PD-L1 (8) by the tumor cells, factors which may also play a role in the local immune response to tumors, all of which have been studied previously in this group of tumors. While there was no association with the macrophage populations, the intraepithelial sub-population of non-macrophage CD14-CD33-CD163+ myeloid cells correlated to strong HLA-class II expression (p=0.005; Table 3).

M1 macrophages form an independent prognostic factor for disease-specific survival

The clinical pathological factors known to influence CxCa prognosis are lymph node status, tumor size, tumor infiltration depth, vasoinvasion and parametria involvement (38-41). To further analyze the correlation between myeloid cells and prognosis, we investigated their correlation with these clinical pathological factors. Intraepithelial CD14+CD33-CD163- myeloid cells (p=0.006), as well as CD14+CD163+ myeloid cells (p=0.005) and specifically the CD14+CD33+CD163+ subpopulation (p=0.007), were associated with lack of metastasis (Table 3). The intraepithelial population of non-macrophage CD14-CD163+ myeloid cells was not correlated to any clinical pathological factors, again indicating that these cells form an unrelated group of tumor-infiltrating myeloid cells.

Because of the relationships found between M1 macrophages, lymph node status and T-cell infiltration, we assessed their prognostic impact in a multivariate analysis. Firstly, stratification of all patients based on the known histopathological risk factors showed that lymph node metastasis (p<0.001), tumor size (p<0.001), infiltration depth (p=0.011) and parametrial involvement (p<0.001) were all associated to a worse disease-specific survival by Cox regression analysis. A multivariate Cox regression analysis of these clinical parameters identified only lymph node status (p=0.004) and tumor size (p<0.001) as independent predictors of disease-specific survival.

Secondly, a Cox regression analysis of the tumor-infiltrating immune cells identified a significant correlation between survival and the total CD14 population (p=0.038), CD14+CD163- cells (p=0.016) and CD14+CD33-CD163- cells (p= 0.007). Finally, the independent clinical pathological risk factors, the number of myeloid cells, as well as the CD8+/ Foxp3+ T-cell ratio were analyzed in a multivariate Cox regression analysis. We confirmed that lymph node status (p<0.001), tumor size (p=0.001) and the CD8+/Foxp3+ intraepithelial T-cell ratio (p=0.015) were all independent survival factors. Furthermore we found that dense M1 macrophages (CD14+CD163-) tumor-epithelial infiltration is an independent survival factor (p=0.011). Analysis with the matured M1 macrophages (CD14+CD33-CD163-) did not reach significance (p=0.053).

Unsupervised clustering based on immune parameters identifies different immune microenvironments associated with survival

This cohort of 86 patients has been extensively studied for the total lymphocyte infiltration, as well as the various lymphocytes sub-populations CD3+CD8-Foxp3-, CD3+CD8-Foxp3+, CD3+CD8+Foxp3-, CD3+CD8+CD57+, CD3-CD8+CD57+, CD3+CD8-CD57+ and CD3-CD8-CD57+ (2;6). Furthermore, this group has been studied for the expression of CXCR7, CXCL12, CXCR4 (32), EGFR (33), MICA (2), HLA-E (9), HLA-G (unpublished), PD-L1 (8) IDO (unpublished), Serpin A1/A3 (31) as well as HLA-class I/II (2;10). To gain a better insight in the immunologic make-up of CxCa, a heatmap of all parameters including the ratio between intraepithelial CD8+ and Foxp3+ T-cells (2) and the intraepithelial myeloid cell populations identified in this study, was constructed. Unsupervised clustering divided the patients into two major groups (Group I and II) that are both subdivided into two subgroups (Ia and Ib, IIa and IIb) and then further into nine smaller subgroups (Figure 3a).

A clear difference between group I and II was the much denser matured M1 macrophage (CD14+CD33-CD163-) infiltration in group II, which co-clustered with different types of immature (CD33+) myeloid cells. Within group I, the tumors of group Ia generally expressed both HLA-class I/II, displayed a strong infiltration of all types of lymphocytes, as well as strong CD33-CD163+ myeloid cells infiltration, irrespective of CD14 expression. Group Ib distinguishes itself from group Ia by a lower lymphocytic infiltrate as well as loss or weak expression of HLA-class I/II in a substantial number of patients. The tumors in group IIa have less intraepithelial T-cell infiltrate and less infiltration of the above mentioned CD33-CD163+ (CD14+ or CD14-) myeloid cells, which are all abundantly present in group IIb. Kaplan-Meier survival curves and log-rank analysis for these groups of patients revealed a significantly better survival for group II ($p=0.045$; Figure 3b), yet no differences were found between subgroups (Ia versus Ib or IIa versus IIb; Supplementary Figure S2b).

Subsequently, survival of the nine small subgroups was analyzed. The clustering of these groups seemed to be based mainly on 4 parameters: HLA-class I/II expression, number of CD8+ T-cells, CD8+/Foxp3+ T-cell ratio and matured M1 macrophages number, indicated by the red boxes in Figure 3a. Subgroup 1 includes tumors with low matured M1 macrophages infiltration and a low CD8+/ Foxp3 T-cell ratio (despite good HLA expression and moderate numbers of CD8+ T-cells). Patients in this group had the worse survival (Figure 3c). Subgroup 2 reflects tumors strongly infiltrated with CD8+ T-cells with a beneficial CD8+/Foxp3+ T-cells ratio, but lacking matured M1 macrophages. This group displayed better survival (Figure 3c). Group 1b (subgroups 3-5) included tumors with low HLA expression, few CD8+ T-cells, a low CD8+/Foxp3+ T-cell ratio and few matured M1 macrophages. The survival in these groups was generally poor (Figure 3c). Group IIb was divided into the small subgroups 7, 8 and 9. They were all abundantly infiltrated with CD14+CD33-CD163- myeloid cells. The main difference was a lower CD8+/Foxp3 intraepithelial T-cell ratio in subgroup 9 and the strongest intraepithelial CD8+ T-cell infiltration in subgroup

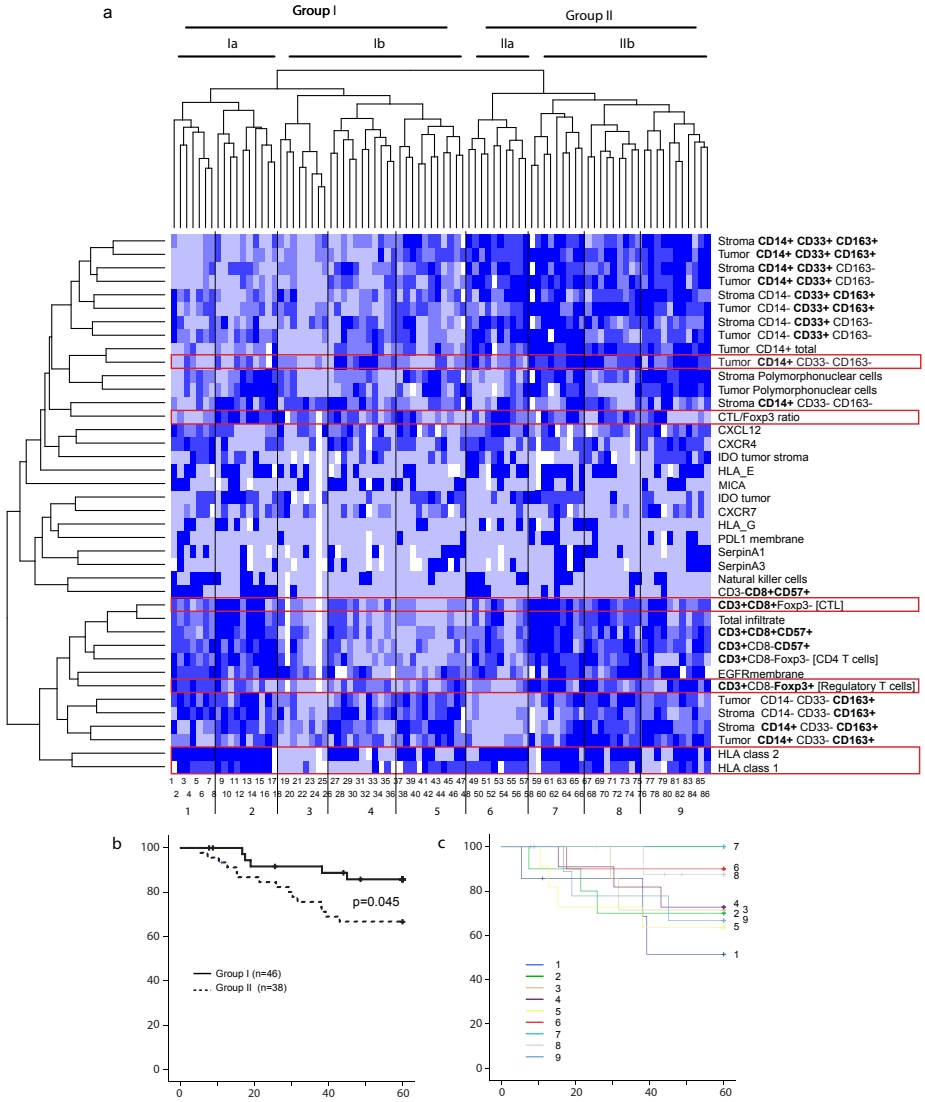

Figure 3. A heatmap was created based on all immunological parameters determined in this patient cohort, followed by unsupervised clustering. The changes from the lowest to the highest quartile are reflected by a darker color, the white boxes are missing data. Along the Y-axis are the 86 patients, with all the immunological parameters indicated to the right. Each column is the unique fingerprint of one patient. The brackets to the left and along the top indicated the unsupervised clustering. Patients are clustered into two groups (Group I and II) based mainly on the CD33+ expressing cells, which co-cluster together with the CD14+CD33-CD163- cells. Both groups can be subdivided into two major groups (Ia, Ib, IIa and IIb) and then further into nine subgroups (indicated along the bottom). The red boxes indicate the parameters which seem important in determining the sub-group clustering. (b) Survival analysis by Kaplan Meijer method and log-rank test of the two major groups, shows a significant disease-specific survival benefit for Group II versus Group I ($p<0.05$). (c) Disease-specific survival analysis by Kaplan Meijer method of all the subgroups 1-9.

7. Analysis of these subgroups revealed that subgroup 9 had the worst survival (similar to patients in subgroups 1-5), whereas patients in subgroup 7 had a 100% 5 year survival (Figure 3c). Subgroup 6 showed a similar survival to patients in subgroup 8, the main difference being the lower numbers of CD8+ T-cells, although a good CD8+/Foxp3 T-cell ratio was maintained.

In conclusion, the unsupervised clustering of patients based on several immune parameters confirmed our earlier observations that M1 macrophages, CD8+ T-cells and Foxp3+ Tregs are tightly associated with the disease-specific survival of CxCa patients.

DISCUSSION

Here we investigated the constitution of tumor-infiltrating myeloid cells in relationship to other tumor-infiltrating immune cells, tumor-characteristics and the disease-specific survival of CxCa patients. Quantification of myeloid cell populations based on the expression of CD14, CD33 and CD163, revealed that a strong intraepithelial infiltration of CD14+ cells, and specifically CD14+CD33-CD163- matured M1 macrophages, is associated with significantly improved disease-specific survival and is an independent prognostic factor as determined by multivariate analysis. The other independent prognostic immune-related factor in CxCa found so far is the intraepithelial CTL/Treg ratio (Figure 2c). Combination of these two factors revealed a substantial increase in survival in the group of patients with tumors displaying dense intraepithelial matured M1 macrophage infiltrate and a high CD8+/Foxp3+ T-cell ratio. Furthermore, immune profiling by unsupervised clustering of 40 different immune parameters revealed a fingerprint that was clearly associated with improved disease-specific survival. The main determinants were the presence of matured M1 macrophages (clustering together with various immature CD33+ myeloid cells) and a high CD8+/Foxp3+ T-cell ratio, both independent prognostic factors. Subgroup analyses provided new insights in the type of immune responses key in the protection against progressive CxCa. We observed that all groups of patients with few M1 macrophages and with low numbers of tumor-infiltrating Tregs (subgroups 2-5), displayed a similar intermediate percentage of disease-specific survival independent of the level of CD8+ T-cell infiltration. This implies that the tumor-infiltrating T-cells are less likely to exert a proper antitumor effect within a tumor microenvironment that does not allow the accumulation of high numbers of M1 macrophages. Indeed in subgroups 6-8, reflecting patients who do very well, the tumors were infiltrated with relatively high numbers of M1 macrophages and displayed a high CD8/Treg ratio (Figure 3).

Previous studies on the number of macrophages in CxCa did not reveal any relationship with clinical parameters (12;19;20), most likely because differences in function were not taken into account. Here, we quantified the number of immature and mature M1 and M2

macrophages. A few recent studies analyzing M2-type macrophages indicated that a dense M2 macrophage infiltration is associated with poor survival in different carcinomas (43-46). In our study, we did not observe this association, finding instead that the number of tumor-infiltrating M1 macrophages was positively associated with survival. Similar observations correlating M1 macrophages to better survival were made in non-small lung cancer (42;43) and breast carcinoma (44).

Studies on colorectal carcinoma also demonstrated that CD14+ TAM were associated with a favorable prognosis and that these CD14+ cells expressed CD40 and often CD80/CD86 (45;46), which can be considered markers of matured antigen presenting cells. In our study, the strongest correlation was found between survival and fully matured (CD33 negative) M1 macrophages, suggesting that the accumulation of matured M1 macrophages reflects a tumor-rejecting microenvironment. The group of M1 macrophages comprised about 25% of cells that expressed CD33+, however, these cells did not provide any disease-specific survival advantage, possibly, because these cells are still not fully activated. Alternatively these CD33+ cells may reflect myeloid derived suppressor cells if they also display a low expression of HLA-DR (14), but this was not tested in our study.

Apart from M2 macrophages a group of myeloid cells was found in vivo that was CD163+ but CD14-. The numbers of these cells did not correlate with the other two myeloid sub-groups, yet correlated to intraepithelial Foxp3+ lymphocytes. Based on our *in vitro* data, showing CD14 expression on monocytes and macrophages, as well as the presence of CD68-CD163+ cells in vivo, we conclude that these non-macrophage CD163+ myeloid cells are likely to reflect immunosuppressive DC's or DC-derived macrophages (29;47).

Our results are important for the development of new strategies to combat cancer. We show that tumor-infiltrating matured M1 macrophages are associated with better survival, irrespective of CD8+ T-cells infiltration. Therapies to block macrophage infiltration – for instance by blocking chemokines or their receptors - thus are not warranted *per definition*. Furthermore, our data suggest that the density of tumor-infiltrating M2 macrophages does not have an impact on overall survival. Therefore, selective inhibition of M2 macrophages without stimulation of M1 macrophages may also prove to be unsuccessful. Therapies should rather aim at reprogramming the abundantly present M2 macrophages towards an M1 phenotype. This can be achieved via multiple pathways (14), including blocking of IL-6 and cyclooxygenase-2 (COX2), both of which are associated with poor survival in CxCa and are known to induce M2 macrophages (21-23), or by using taxoids (48). Activation of the tumoricidal function of M1 macrophages can be achieved through ligation of CD40. In pancreatic ductal adenocarcinoma combining gemcitabine and agonistic CD40-antibody induced tumor regression due to the activation of tumoricidal macrophages and subsequent depletion of tumor stroma (49). Here, the myeloid derived suppressor cells (MDSC) were probably effectively removed by the use of gemcitabine (50). Notably, CD4+ type 1 helper T-cells are well equipped to reprogram CD14+CD163+ macrophages towards activated M1

macrophages via CD40-CD40L interaction and the production of Interferon (IFN)γ (23). Our observations that patients whose tumors are infiltrated with a dense T-cell population comprising relatively low numbers of Foxp3 regulatory T-cells and a dense population of mature CD14+CD163- myeloid cells, have the best clinical performance, suggest that these two populations collaborate to resist tumor cells. These results should be validated by prospective trials. Current immunotherapy trials aim at reinforcing the tumor-specific T-cell response to CxCa, but our data argue that these therapies are most likely to have success if the tumors are infiltrated with matured M1 macrophages. Therefore, pre-selection of patients based on dense CD14+ cells infiltration may help to improve success rates. However, therapy combining the induction of tumor-specific IFNγ-producing CD4+ T-helper cell and cytotoxic CD8+ T-cell responses, the attraction of M1 macrophages or reprogramming of resident myeloid cells, may be beneficial to a larger group of patients.

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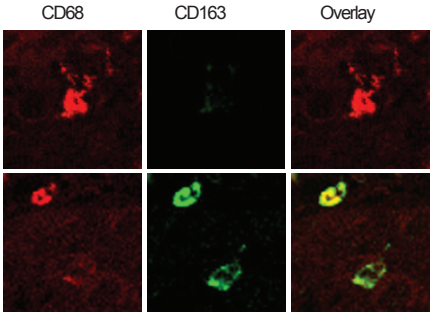
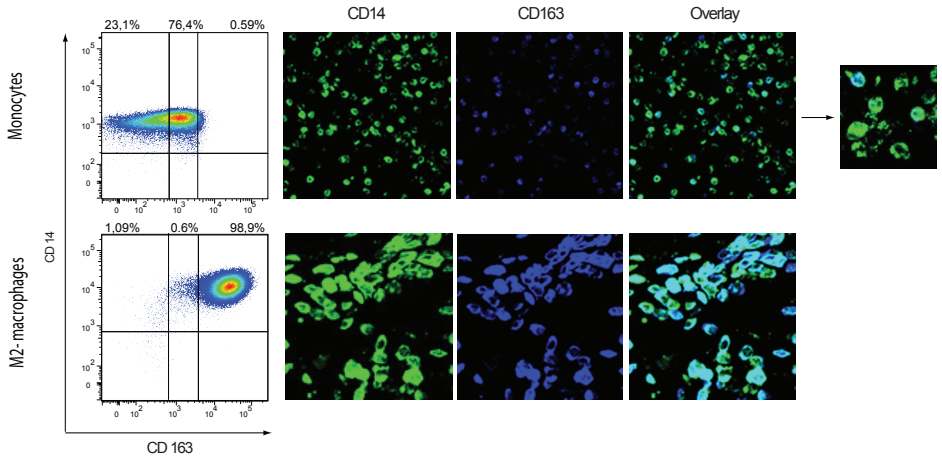
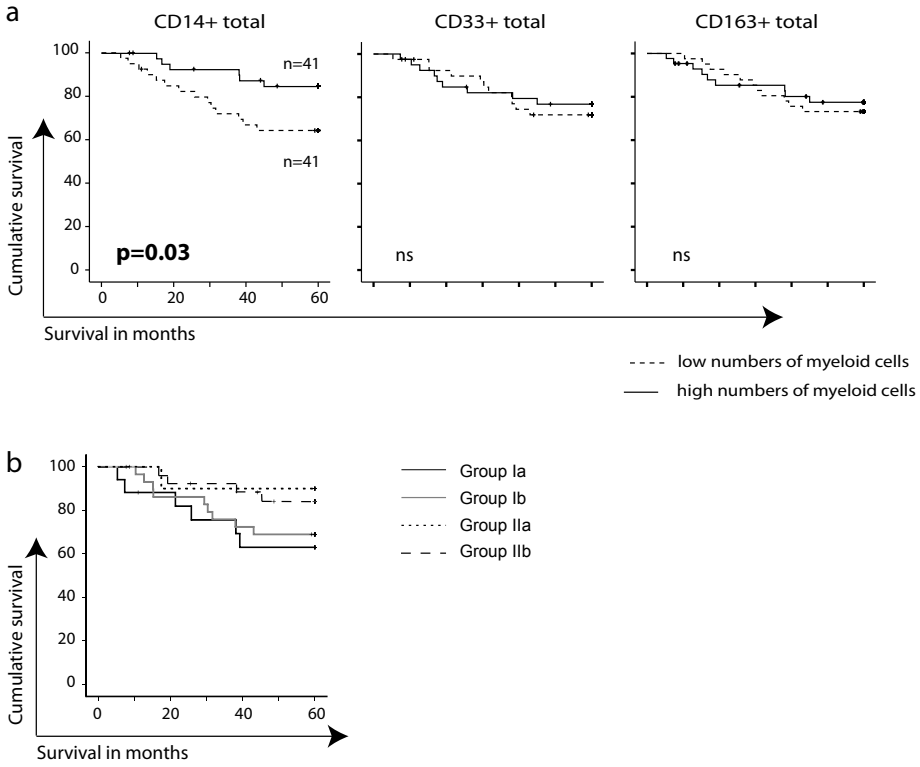


Supporting information 1. Immunohistochemical staining for CD68 and CD163 showing CD68+CD163- single staining, double staining CD68+CD163+ and CD68-CD163+ single staining.



Supporting information 2. 76% of freshly isolated monocytes show intermediate expression of CD163, while cultured M2 macrophages express up to 1000x more. Immunohistofluorescence staining of monocytes shows mainly CD14+CD163- (green) cells, whole most of the M2 cells were positive for both markers (CD14+CD163+, turquoise).



Supporting information 3. (a) Kaplan Meijer curves of intraepithelial myeloid cell populations based on the total CD14+, CD33+ and CD163+ cells shows a significant survival benefit for the total CD14+ cells. (b) Survival curves of the different subgroups of patients (Ia, Ib, IIa and IIb) created in the heatmap revealed no significant differences.