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Original article

# Myeloid-derived suppressor cells reveal radioprotective properties through arginase-induced L-arginine depletion

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ABSTRACT

*Background and purpose:* High arginase-1 (Arg) expression by myeloid-derived suppressor cells (MDSC) is known to inhibit antitumor T-cell responses through depletion of L-arginine. We have previously shown that nitric oxide (NO), an immune mediator produced from L-arginine, is a potent radiosensitizer of hypoxic tumor cells. This study therefore examines whether $Arg^+$ overexpressing MDSC may confer radioresistance through depleting the substrate for NO synthesis.

*Material and methods:* MDSC and Arg expression were studied in preclinical mouse CT26 and 4T1 tumor models and further validated in rectal cancer patients in comparison with healthy donors. The radioprotective effect of MDSC was analyzed in hypoxic tumor cells with regard to L-arginine depletion.

*Results:* In both mouse tumors and cancer patients, MDSC expansion was associated with Arg activation causing accelerated L-arginine consumption. L-Arginine depletion in turn profoundly suppressed the capacity of classically activated macrophages to synthesize NO resulting in impaired tumor cell radiosensitivity. In advanced cT3-4 rectal cancer, circulating neutrophils revealed Arg overexpression approaching that in MDSC, therefore mounting a protumor compartment wherein $Arg^+$ neutrophils increased from 17% to over 90%.

*Conclusions:* Protumor $Arg^+$ MDSC reveal a unique ability to radioprotect tumor cells through L-arginine depletion, a common mechanism behind both T-cell and macrophage inhibition.



The prognosis for locally advanced rectal cancer (RC) is mainly based on TNM classification that underscores the metastatic tumor spread at the time of diagnosis. However, the predictive accuracy of this staging approach is limited, since it relies on the perception that the tumor response is solely dependent on the genetic signature of tumor cells with no attention to the host immune system [1].

With recent advances in immunological profiling of both local and systemic immunity, the beneficial effect of tumor-infiltrating lymphocytes (TIL), as opposed to protumor myeloid-derived suppressor cells (MDSC), has been highlighted [2–5]. This led to an appreciation of the tumor immune landscape and the introduction of prognostic immunoscores, like the Glasgow microenvironment and TIL immunoscores, assessed in surgical tumor specimens [6–10]. In daily practice, the blood 'neutrophil-to-lymphocyte ratio' (NLR) can be used to estimate tumor-associated inflammation that drives the expansion of protumor N2-type neutrophils (comprising MDSC) at the cost of potentially antitumor Th1-type lymphocytes. An increased NLR is reported to be a strong indicator of poor outcome in RC patients following the resection of the primary tumor and is recommended for risk stratification in chemoradiotherapy [11,12].

While the prognostic importance of immune cells is well accepted, their impact on tumor cell radiosensitivity has emerged only recently. Our previous studies demonstrated that classically activated macrophages may radiosensitize by-standing colorectal cancer cells through the production of nitric oxide (NO) from L-arginine by inducible NO synthase (iNOS), the key marker of antitumor M1 polarization within the macrophage/monocyte lineage [13,14]. In addition, activated $CD8^+$ lymphocytes can produce IFN-γ at levels sufficient to trigger the iNOS/NO pathway in tumor cells and macrophages [15]. The principal mechanism of radiosensitization under hypoxic (but not normoxic) conditions is linked to the intrinsic ability of NO to down-regulate mitochondrial respiration, resulting in oxygen sparing and tumor cell re-oxygenation

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[14]. Thus, our data prompt that the T-cell/macrophage axis is an important determinant of enhanced hypoxic radiosensitivity dependent on the bioavailability of L-arginine. In contrast, the radiobiological effects of MDSC/neutrophils remain poorly understood.

Remarkably, L-arginine plays also a critical role in antitumor immunity by sustaining T-cell proliferation and their zeta-chain receptor expression [16–18] Indeed, L-arginine depletion by MDSC, which overexpress arginase-1 (Arg), is known to contribute to tumor-associated immunosuppression by inducing T-cell anergy. Since L-arginine appears to be instrumental in both adaptive immune responses and NO-mediated radiosensitization, we have hypothesized that $Arg^+$ MDSC may provoke radioprotection through the same mechanism of accelerated L-arginine consumption [19]. In our vision, novel immune biomarkers that predict the risk of radiotherapy failure are urgently needed, as a further escalation of radiation dose delivered by intensity-modulated radiotherapy plus a simultaneous integrated boost does not improve overall survival in RC [20]. With this in mind, the present preclinical/translational study was designed to provide a proof-of-concept on the potential of MDSC to deplete L-arginine and thereby to suppress the radiosensitizing nature of M1 macrophages. Next, we performed a detailed immunological and functional profiling of $Arg^+$ MDSC /neutrophils in RC patients in comparison with healthy donors.

## Materials and methods

### *Cell lines and chemicals*

Mouse macrophage RAW264.7, colon CT26 and mammary 4T1, and human colon HCT116 tumor cell lines were obtained from American Type Culture Collection (ATCC, Manassas, USA). All experiments were performed in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% bovine calf serum (Greiner Bio-one, Belgium). Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

### *Human MDSC*

Peripheral blood was collected from 25 patients with locally advanced T3-4 RC and 15 healthy volunteers. Neutrophils were isolated by the "Whole blood anti-CD15 MicroBead kit" (Miltenyi biotec, Bergisch Gladbach, Germany), and phenotyped by flow cytometry as $Lin^-HLA^-DR^{low}CD33^+CD15^+CD16^+$ and MDSC were defined as their $CD16^{low}$ subset [21,22]. The following antibodies were used: HLA-DR, CD14, CD15, CD19, CD20, CD56 (eBioscience Frankfurt, Germany) CD3, CD4, CD8, CD16, CD33 (Miltenyi biotec), and Arg (R&Dsytems, Minneapolis, USA).

### *Mouse MDSC*

Intramuscular (IM) CT26 and 4T1 tumors were grown for 14 days in Balb/c mice (Charles River Laboratories, Brussels, Belgium) following injection of 0.5 x $10^6$ cells, and MDSC were isolated from the tumor and spleen by anti-CD11b Microbeads (Miltenyi biotech) as previously described [23]. Freshly isolated $CD11b^+$ cells were further conditioned for 24 h *in vitro* in normoxia or hypoxia (21 or 1% oxygen), or intraperitoneally (*in vivo*) in tumor-bearing mice, as adapted after Corzo et al. [24]. MDSC were phenotyped as $CD11b^+$ (eBioscience) and $Gr\text{-}1^+$ (Biolegend, Fell, Germany), and further subdivided into $Ly6G^+Ly6C^{low}$ granulocytes and $Ly6G^-Ly6C^{high}$ monocytes [4]. MDSC-conditioned medium (supernatant) was prepared at a density of $10 \times 10^6$ cells/ml during 24 h, aiming at depleting L-arginine supplied at 600 μM.

### *Activation of immune cells*

Peritoneal macrophages from Balb/c mice were polarized to M1 and M2 types by exposure for 16 h to LPS (0.1 μg/mL)/IFN-γ (10 ng/mL) and IL-4 (10 ng/ml) respectively. Either fresh or conditioned medium from $Arg^+$ MDSC was used during macrophage activation. T cells were isolated by anti-CD3 MicroBeads (Miltenyi biotec) and stimulated using anti-CD3/CD28 antibodies (BD Biosciences, Erembodegem, Belgium) at 3 μg/ml to produce conditioned medium enriched in IFN-γ. The proliferation index was assessed using T cells that were labeled with 0.5 μM CFSE (carboxyfluorescein succinimidyl ester) and stimulated with anti-CD3/CD28 Dynabeads® (Life technologies, US), while IFN-γ secretion was assessed by ELISA (Thermo Fischer scientific Inc., IL, USA).

### *Arg and iNOS profiling*

Arg expression in immune cells was assessed by flow cytometry and quantitative RT-PCR, using the specific primers Mm01190441_g1 (Thermo Fischer Scientific). Arg and iNOS activities was measured respectively through urea production (Bio assay systems USA and Immundiagnostik Germany) and by Griess assay that quantifies nitrite, an oxidative NO product [13].

### *Hypoxic radioresponse*

The hypoxic tissue-mimetic co-culture system (TMCS) has been described in detail elsewhere [14]. Briefly, monolayers of tumor cells and activated macrophages were subjected to metabolic hypoxia for 45 min, and irradiated at a dose rate of 2 Gy/min on a 6MV Linac. Survival fractions were measured by an 8-day clonogenic assay, based on at least 50 cells per colony [14]. Oxygen consumption in TMCS was monitored by a MitoXpress probe (Luxcel Biosciences, Cork, Ireland). When indicated, conditioned medium from $Arg^+$ MDSC or activated T cells was used during macrophage activation. Aminoguanidine (AG) was added at 1 mM to block iNOS as indicated.

### *Statistics*

All assays were repeated at least three times. One- or two-way ANOVA followed by Bonferroni's multiple comparison or t-test were performed using prism 6.1 (Graphpad, USA, CA). The number of asterisks in the figures indicates the level of statistical significance as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

## Results

### *MDSC immunoprofiling and conditioning in mouse tumor models*

Fourteen days following IM implantation of CT26 and 4T1 tumors, we observed a significant expansion of $CD11b^+Gr\text{-}1^+$ MDSC, consisting of $Ly\text{-}6C^+$ monocytic (Mo) and $Ly\text{-}6G^+$ granulocytic (Gr) subpopulations (Fig. 1A and C). Remarkably, splenic MDSC displayed only marginal Arg expression (below 7%) compared with those in the tumor (over 45%), as detailed in Fig. 1B. Since MDSC are defined by function rather than phenotype, we additionally examined their immunosuppressive activity (Fig. 1D). As expected, MDSC inhibited T-cell proliferation by 4-5 times (at a 1:1 ratio) while IFN-γ production by T cells was suppressed by 3-fold.

Despite that MDSC levels within CT26 and 4T1 tumors reached respectively 45 and 87% of $CD45^+$ leukocytes, their isolation in numbers sufficient for radiobiological applications remained problematic while freshly isolated splenic MDSC lacked Arg. Therefore, we examined the possibility to condition/differentiate splenic MDSC in normoxic or hypoxic cultures (*in vitro*) and in the peri-

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