

Smoothing T cell roads to the tumor

Chemokine post-translational regulation

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We described a novel tumor-associated immunosuppressive mechanism based on post-translational modifications of chemokines by reactive nitrogen species (RNS). To overcome tumor immunosuppressive hindrances, we designed and developed a new drug, AT38, that inhibits RNS generation at the tumor site. Combinatorial approaches with AT38 boost the effectiveness of cancer immunotherapy protocols.

The paradigm of cancer immunoediting is an emerging cornerstone of current tumor immunology. Tumors established various overlapping mechanisms to escape from immunosurveillance by sculpting host immune responses and angiogenesis.^{1,2} Tumor-induced T-cell tolerance is one of the major hurdles (primarily exerted by regulatory lymphoid and myeloid cells) that favors cancer progression and spreading. Successful localization of tumor-specific cytotoxic T cells (CTLs) is widely recognized as a crucial determinant of tumor immunity. The prognostic value of tumor-infiltrating lymphocyte (TIL) presence within the tumor mass has been advanced by a number of studies in the last years. However, this pioneer concept was definitively strengthened by the finding that high numbers of cytotoxic and memory T cells in human colorectal cancer patient biopsies is predictive of positive prognosis, independently from cancer stage.³ Among the paralyzing factors circumventing T cell access to the tumor, we previously indicated the intratumoral generation of reactive nitrogen species (RNS) through a deregulated metabolism of L-arginine by arginase and nitric oxide synthase (NOS).⁴

It was recently documented how nitrate stress directly affects T-cell signaling molecules leading to T-lymphocyte

dysfunction.^{5,6} Nitrotyrosine detection represents a trustworthy marker of the in situ RNS release and biological activity. In human cancer, the “nitro proteome” extent is quite heterogeneous. While examining the occurrence of this phenomenon in various human tumors, we repeatedly observed an opposite correlation between nitrotyrosine staining and T cell positioning within primary tumor lesions. As tuners of cell migration, chemokine networks are frequently affected by cancer cells to promote tumor growth and spreading. We speculated that CCL2, an inflammatory chemokine fostering both CTL and myeloid cell recruitment to tumors, could be modified by RNS assault. To detect directly the presence of the RNS-modified chemokine in cancer specimens, we isolated a single-domain recombinant antibody from a llama naïve library, which specifically interacts with the nitrosylated/nitrated CCL2 (N-CCL2). By the use of this new reagent, we demonstrated the presence of N-CCL2 in human prostate and colon carcinomas, and found that it directly correlates with the intratumoral nitrotyrosine staining. In the same tumors, CTLs were gathered at the periphery, outside the areas positive for nitrotyrosine, suggesting that N-CCL2 was not effective in recruiting T cells. This hypothesis was

supported by in vitro experiments highlighting an almost completely defective T lymphocyte migration in response to N-CCL2 stimulation. Conversely, myeloid cells retained their ability to sense N-CCL2 gradients. This antithetical behavior, likely dependent on the lower expression of CCL2 receptor (CCR2) in T cells compared with myeloid cells, could account for the intratumor preferential accumulation of the myeloid subset, including tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), instead of tumor-antigen specific CTLs. As extensively reported, tumor-recruited MDSCs contribute to RNS generation⁴ feeding the vicious circle leading to N-CCL2 formation.

Intravital imaging studies in mouse models have unraveled T cell leading paths within the tumor environment.^{7,8} Investigating by two photon microscopy TIL mobility within TC-1 lung epithelial tumors, Mrass and colleagues concluded that antigen recognition was a critical determinant of T-cell migration within tumors.⁷ In the absence of recognized road signs, however, antigen-experienced CTLs failed to infiltrate tumor primary lesion, gathering at the periphery. Indeed, RNS appear to block T-cell entrance to the tumor mass exploiting a post-translational masking of chemokines. We documented

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the concomitant expression of nitrotyrosine and N-CCL2 in different mouse tumor models. While considering localization in these tumors, we confirmed a preferential distribution of T cells at the periphery of the neoplastic lesions. Even adoptively transferred OT-1 cells, bearing an high avidity TCR for ovalbumin (OVA), congregated at tumor surrounding stroma in EG-7-OVA tumors and did not efficiently infiltrate the tumor core.

RNS generation thus prevents an effective CTL recruitment to the tumor by raising a tumor-related chemical barrier. Previous studies suggested that blocking RNS production could restore T cell responsiveness and improve antitumor responses.^{9,10} Reasoning on these preliminary findings, we designed and developed a new compound - AT38 - that efficiently interferes with RNS generation by modulating ARG and NOS enzyme expression.

A time-scheduled administration of AT38 in tumor-bearing mice caused a reduction in nitrotyrosine formation and the subsequent unmasking of TIL chemoattractant signals. Given these promising results, we tested AT38 in combination with passive immunotherapy. Adoptive cell therapy (ACT) for cancer is one of the most promising immunotherapy approaches; nevertheless current ACT protocols are limited by a number of variables. In both

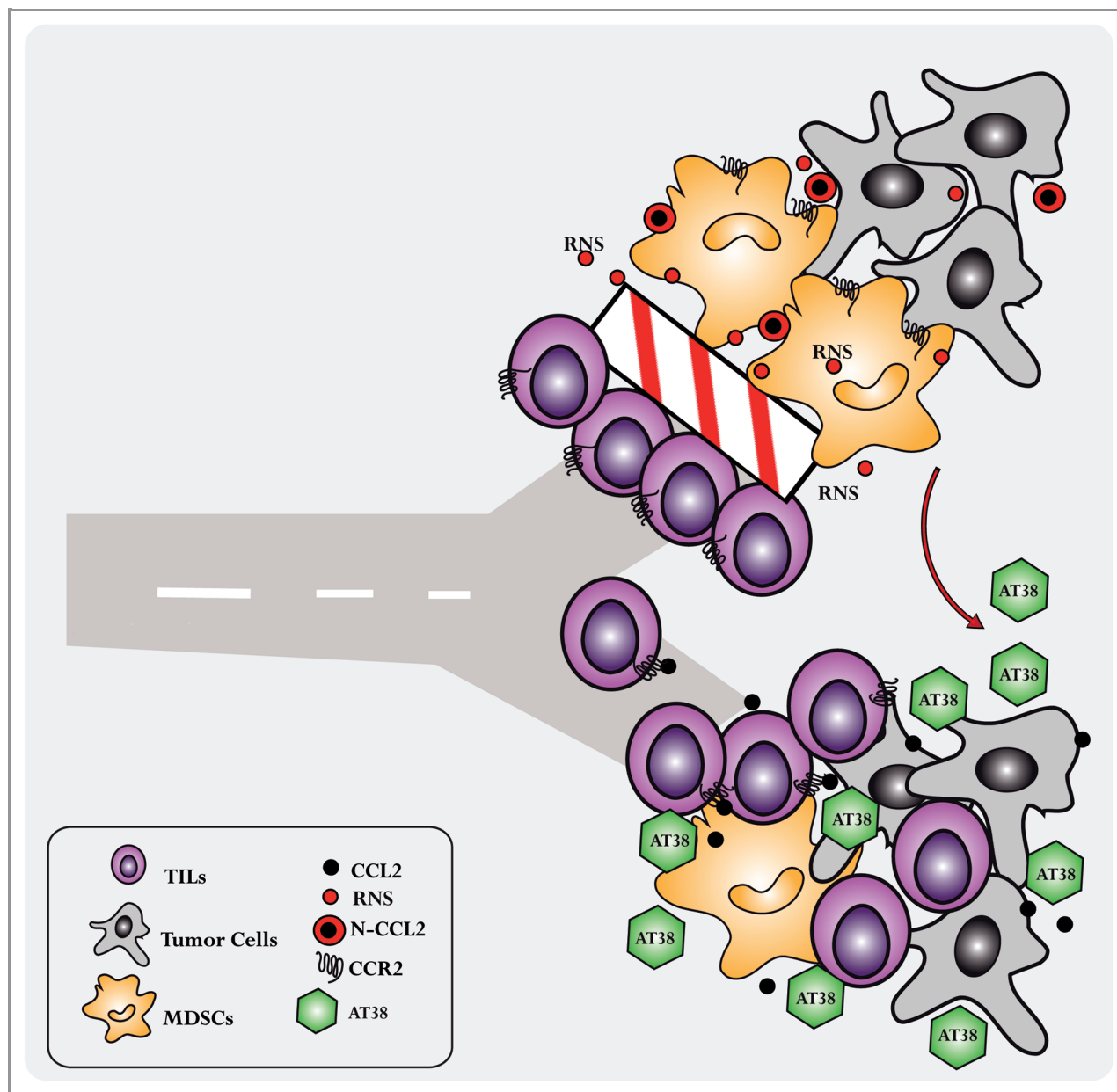


Figure 1. RNS which are generated at a constant rate within the tumor mass stably modify CCL2. N-CCL2 is a poor chemoattractant for T cells. Conversely MDSCs, which express high CCR2 levels, retain the ability to sense N-CCL2 gradients. In this scenario, insensitive CTLs are trapped at the periphery of the tumor, while receptive MDSCs accumulate within the tumor mass sustaining cancer growth. AT38 blocks RNS production and overcomes tumor-dependent immunosuppressive constraints promoting recruitment of TILs to the tumor where they can exert their killing activity.

EG-7 thymoma and MCA-203 fibrosarcoma models, the combination of AT38 with ACT protocols allowed the adoptively transferred, tumor-specific CTLs to migrate properly to the tumor core and promote tumor rejection. AT38 might have multiple effects, for example, it could increase CTL recognition of tumor cells that are rendered defective in presentation of their tumor antigens through Class I

MHC molecule by nitritative stress² and decrease the immunosuppressive activity of MDSCs within the tumor environment.¹⁰ Certainly, all these activity might contribute to the adjuvant action on ACT (Fig. 1). However, direct effect on CCL2 unmasking seems to be an absolute requirement since *ccr2*^{-/-} T lymphocytes are still kept at the periphery of the tumor whereas a significant recruitment

of TILs was achieved when native, unmodified CCL2 was inoculated directly within untreated tumors. Despite the plethora of chemokine receptors able to direct T lymphocytes, CCR2 is thus essential to allow the completion of trafficking process within the tumor mass, suggesting the existence of an unexpected territorial diversity within different tumor districts.

References

1. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang LP, et al. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. *Nature* 2011; 475:226-30; PMID:21753853; <http://dx.doi.org/10.1038/nature10169>
2. Lu T, Ramakrishnan R, Altioek S, Youn JI, Cheng P, Celis E, et al. Tumor-infiltrating myeloid cells induce tumor cell resistance to cytotoxic T cells in mice. *J Clin Invest* 2011; 121:4015-29; PMID:21911941; <http://dx.doi.org/10.1172/JCI45862>
3. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; 313:1960-4.
4. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nature* 2005; 5:641-54.
5. Kasic T, Colombo P, Soldani C, Wang CM, Miranda E, Roncalli M, et al. Modulation of human T-cell functions by reactive nitrogen species. *Eur J Immunol* 2011; 41:1843-9; PMID:21480210; <http://dx.doi.org/10.1002/eji.201040868>
6. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* 2007; 13:828-35; PMID:17603493; <http://dx.doi.org/10.1038/nm1609>
7. Mrass P, Takano H, Ng LG, Daxini S, Lasaro MO, Iparraguirre A, et al. Random migration precedes stable target cell interactions of tumor-infiltrating T cells. *J Exp Med* 2006; 203:2749-61; PMID:17116735; <http://dx.doi.org/10.1084/jem.20060710>
8. Schaer DA, Li Y, Merghoub T, Rizzuto GA, Shemesh A, Cohen AD, et al. Detection of intra-tumor self antigen recognition during melanoma tumor progression in mice using advanced multimode confocal/two photon microscope. *PLoS ONE* 2011; 6:e21214; PMID:21731676; <http://dx.doi.org/10.1371/journal.pone.0021214>
9. Bronte V, Kasic T, Gri G, Gallana K, Borsellino G, Marigo I, et al. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. *J Exp Med* 2005; 201:1257-68; PMID:15824085; <http://dx.doi.org/10.1084/jem.20042028>
10. De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, Del Soldato P, et al. Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. *Proc Natl Acad Sci USA* 2005; 102:4185-90; PMID:15753302; <http://dx.doi.org/10.1073/pnas.0409783102>