Original Article

Spleen migrating dendritic cells primed with CC531 colon cancer antigen and LPS – is it a method to compromise liver metastases?

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ABSTRACT

Background: One of the flaws of anti-tumor vaccination is that in vitro primed DC administered iv reach liver metastases only in a few percent and it is not possible to administer them continuously. Therefore, it seemed rational to create a model with vaccination performed in vivo with the specifically primed DC and cytotoxic T lymphocytes continuously supplied to liver vascular bed.

Aim: The question we raised was whether anti-tumor immunized splenic DC flowing to liver metastases could adhere to and be cytotoxic to tumor cells.

Methods: We immunized rats with CC531 tumor cells and stimulated them with E. coli LPS. Subsequently, spleen DC-enriched population was isolated, its activation by LPS and cytotoxicity against CC531 cells were measured. Spleen cells flowing via splenic vein home to the liver. They can be retrieved by simple wash-out as liver sinusoidal wash-out cells (LSWC). Their cytotoxicity against CC531 cells was evaluated. Moreover, in vitro adherence of splenic DC-enriched cells and LSWC to CC531 liver metastases was measured. Results. In vivo immunization of splenic population containing DC, NK cells and lymphocytes with CC531 cells and stimulation with LPS activated these cells but did not increase the cytotoxicity against CC531 cells. There was also no increase in cytotoxicity of LSWC. Adhesion of splenic DC and LWSC to liver CC531 metastases on cryosections was higher than to the adjacent liver tissue. However, it was more expressed on tumor stromal than neoplastic cells. The level of splenic Treg cells down-regulating immune response was found only slightly increased after immunization. This suggests that Treg do not participate in down-regulation of anti-tumor suppressive process.

Conclusions: In the model of in vivo immunization against CC531 cells, low level of spleen DC and spleen-derived LSWC cytotoxicity as well as adherence rate to tumor cells were observed.

KeyWords: spleen, dendritic cells, colon cancer, metastasis, vaccines

Introduction

Liver metastases of colon cancer assessed immunohistochemically are characterized by limited cellular infiltrates around the tumor tissue, low number of apoptotic cancer cells but high number of apoptotic tumor surrounding mononuclear cells. In spite of visible host cellular reaction metastatic tumor expands rapidly. Present in liver sinusoids, specific for this organ CD3+CD4-CD8+, CD3+CD4-CD8- and CD3+CD56+ TCRαβ lymphocytes reveal cytotoxic activity against certain tumor lines but their clinical effect is slight (1, 2, 3).

Recently, attention has been focused on dendritic cells (DC), which a) are an integral part of nonparenchymal fraction of liver cells, b) are present in the spleen and colonize liver via splenic vein, c) participate in internalization of fragments of tumor apoptotic cells, d) process and present tumor antigens to T lymphocytes. Thus, a concept aroused of application in cancer therapy of DC specifically activated against tumor associated antigen (TAA) as the so called "tumor vaccination" (4-8). DC priming is carried out using a number of natural and processed molecular structures (9,10).

Anti-tumor vaccination with tumor antigens is done ex vivo. It contains the following flaws: a) in vitro primed DC administered iv only in a few percent reach the cancer focus, b) administered in the region of the liver metastases result in embolization of blood vessels, therefore cannot be dispersed in the tumor mass, c) there are

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no supporting stroma cells in the vicinity allowing the DC to function, d) it is vital to administer them regularly. For this reason it seems rational to create a model in which the vaccination was performed in vivo and the specifically primed DC and cytotoxic T lymphocytes could be continuously supplied into the liver vascular bed. This can be achieved by mobilizing immunized anti-tumor spleen migrating populations. Spleen contains DC and T and B lymphocytes forming functional units where priming by bacterial and tumor antigens takes place. Spleen antigen-pulsed DC are potent stimulators of antigen-specific lymphocyte proliferation and contain potent regulatory cells (11).

Interestingly, metastases and primary tumors are rarely seen in spleen and its neoplastic involvement ranges only around 2% (12, 13). This may suggest the presence in the spleen of cell populations highly effective in tumor antigen elimination (14).Stimulation of splenic DC with bacterial LPS may enhance this process (15).

In this study we investigated whether the DC-enriched population of splenocytes and their fraction migrating to the liver may be effectively in vivo stimulated with CC531 antigen and become cytotoxic. As migrating spleen cells are transported via splenic vein to the liver, liver sinusoidal wash-out cells (LSWC) were isolated and their cytotoxicity against CC531 cells was measured. Moreover, the in vitro adherence of splenic DC-enriched cells and LSWC to CC531 liver metastases was evaluated.

Immunization with CC531 cells and stimulation with LPS of splenic population containing DC, NK cells and lymphocytes acti vated these cells but did not increase the specific cytotoxicity a gainst CC531 cells. This was proved both in the in vitro DC-CC531 cluster formation and cytotoxic tests. Adhesion of splenic DC to liver CC531 metastases on cryosections was higher than to the adjacent liver tissue, however, it was more expressed on tumor stromal than neoplastic cells. The level of splenic Treg cells down-regulating immune response was found only slightly in creased after immunization . Taken together, low level of spleen cellular reactivity to tumor in the in vitro direct cluster formation between spleen effector and CC531 cells and cytotoxicity as well as adherence to tumor metastases were observed despite of anti-tu mor immunization with LPS boosting.

Material and Methods:

Animals

Wistar (W/Wag) (RT1a) rats weighing 200-300 g, from our ani mal husbandry, were used. Animals were treated humanely using approved procedures in accordance with the guidelines of the Insti tutional Animal Use and Care Committee. Consent of Ethics Com mittee on Animal Experimentation was obtained.

CC531 tumor cells

CC531 is an adenocarcinoma cell line, originating in the colon of Wag/Rij rats exposed to methylazoxymethanol. CC531 cells were cultured in alpha-RPMI (RPMI 1640 medium, supplemented with 2 mM L-glutamine, 10% heat-inactivated foetal calf serum (FCS; Gibco, Bio-Cult, Irvine, Scotland), 50 micromolars beta-mercapto-ethanol, 100 U/ml penicillin and 100 micrograms/ml streptomycin). Cells were detached with trypsin-EDTA solution at 37° C. Viability of cells was assessed by trypan blue exclusion and was always > 95%.

Generating CC531 metastases to the liver

CC531 cells in suspension 106 cells per 0,5 ml Hanks solution were injected into the mesenteric vein. Metastases were observed after 14 to 21 days.

Immunization with CC531 cells

Rats were given with 2x106 CC531 cells or their debris with Freund adjuvant intraperitonealy. CC531 administration was re peated without adjuvant after 7 days. Then, serum was collected and humoral cytotoxicity was measured.

Assessment of leukocyte migration from spleen into liver

Splenocytes were loaded with 1 um diameter FITC stained microspheres (Invitrogen, Eugene, USA). Spleen pedicle was clamped and cells were injected under the capsule. After 3 minutes the clamp was released, fragments of liver tissue were collected and frozen at -70C. Slides 3 um thick were evaluated under the fluorescent microscope.

Splenic DC isolation and culture (16, 17)

Fragments of spleen from control and CC531 immunized rats were digested using collagenase D (Sigma, Steinheim, Germany) and spun down for 10 min at 280g at 4° C. The cell pellet was re suspended in 5 ml of dense BSA (Sigma, Steinheim, Germany), overlayed with 1,5 ml of RPMI-1640 (Life Technologies Ltd, Paisley, United Kingdom) and centrifuged for 15 min at 6000g at 4° C (step I). Cells from the interface region were collected. As the next step, the pellet was resuspended in RPMI -5 to a concentration of 107 cells/ml, plated in a volume of 4ml on plastic dish and incubat ed for 90 min. The nonadherent cells were removed by gentle washing with RPMI-1640. The medium was replaced by RPMI-5 and cells were incubated for 20h (step II). Then, cells were harvest ed, centrifuged and pellet was resuspended in 1 ml fresh RPMI-5 medium and 50 sheep erythrocytes coated with rabbit anti-erythro cyte serum (Sigma, Steinheim, Germany) per leukocyte were added, centrifuged for 10 min at 70g at 4° C and without aspirating the supernatant was placed on ice for 30 min. The cell suspension was overlayed on 5 ml dense BSA. The interface cells were collected. Such suspension yielded 6-9x105 cells per spleen (step III).

The population from step III contained DC and some lymphocytes.

Flow-cytometric assessment of DC-enriched splenic leukocyte population

For identification of cellular antigens direct immunofluorescence staining method was applied using specific mouse monoclonal antibodies and immunoglobulins for isotypic controls (Serotek, UK). Cells (106) in 100μ l PBS + 2% FCS were incubated for 30 min at +4 ℃ in the presence of 10μ l of monoclonal antibody conjugated with phycoerythrine (Table 1). Analysis was performed in flow cytometer FACStar (Becton & Dickinson, USA) and statistical analysis was carried out using FlowMATE computer software (Dako, Denmark).

Mononuclear cells isolation from peripheral blood, spleen and liver sinusoids

Peripheral blood mononuclear cells (PBM) and liver sinusoidal washout cells (LSWC) were isolated from normal and immunized rats. LSWC were obtained by flushing livers through the portal

Table 1 Characteristics of applied antibodies

Antibody specific for:	Clone	Dilution	Target cells
MHCII *	MRC OX-6	1:1a00	Class II antigens, macrophages, dendritic cells, B lymphocytes
CD43*	W3/13	1:100	T lymphocytes, thymocytes, plasmatic cells
CD 8	OX8	1: 100	T cytotoxic lymphocytes
CD 4	W3/25	1:100	T helper lymphocytes
CD25	OX39		IL2 receptor
CD68*	ED1	1:100	Macrophages, monocytes
CD103*	OX-62	1:30	Migrating dendritic cells, some T lymphocytes
CD161a (NKR-P1A)**	10/78	1:100	NK cells, dendritic cells
PAN B Cell Marker*	RLN-9D3	1:100	B lymphocytes
OX12 (anti rat kappa light chain)*	MRC OX-12	1:100	Lymphocytes (kappa light chain), mainly B lymphocytes
Antibody specific for: * Producer:	Clone	Dilution	WESTERN-BLOTTING Proteins characteristic for:
TLR2***	H-175	1:1000	Human, mouse or rat TLR2 expressed mainly on blood lymphocytes, macrophages and monocytes
TLR4***	H-80	1:1000	As above
TLR9***	C-20	1:1000	Human, mouse or rat TLR9 expressed on spleen, lymph nodes, bone marrow cells and blood lymphocytes
HSP60***	H-300	1:1000	Human, mouse or rat HSP60
HSP90***	H-114	1:1000	Human, mouse or rat Hsp 90α (HSP 86) and HSP 90β (HSP84)

Producer : *– Serotec, **– PharMingen, ***– Santa Cruz Biotechnology

vein with Hanks solution. The effluent fluid was collected.

LPS activation of DC–enriched splenic leukocyte population.

E.coli 0127:B8 lipopolisacharide (LPS) (Sigma, Steinheim, Germany) was used. For in vivo stimulation 1 mg/ml of LPS was given intraperitoneally for 6 consecutive days.

Western–blotting assessment of proteins produced by stimulated splenic leukocyte population

Monoclonal antibodies against TLR 2, 4 and 9, HSP 60 and 90 were applied. Densitometric protein bands were assessed using easyDens software (Cortex Nova, Bydgoszcz, Poland). Total band density was calculated as sum of pixels intensity minus liminal value.

Measuring of cytokines produced by stimulated splenocytes.

ELISA was performed for IL-12 and IFN-γ using commercial kits (Rat Il-12p70 Immunoassay Kit, Biosource, Belgium; Rat IFN-γ Immunoassay Kit, R&D System Europe, UK).

Rosette formation test between CC531 and DC–enriched splenic population.

CC531 cells were incubated with DC-enriched splenic leukocytes population or PBM or LSWC at E/T 1:10 ratio. Reaction was observed under confocal microscope following staining of effector cells with monoclonal antibody against MHC classII(OX6) , CD43 (W3/13), CD14 (ED1), OX62 and CD161a (NK). As a rosette was considered a cluster of CC531cell with at least one effector cell. Twenty microscopical fields were evaluated.

Cytotoxicity assay in suspension of DC–enriched splenic population and CC531.

CC351 cells at a concentration of 1x106 were labeled with carboxyfluorescein (CFDA, Sigma, Steinheim, Germany), mixed with effector cells at E/T ratios 40:1, 20:1, 10:1, and incubated for 24h. The percentage of cytotoxicity was assessed using FACScan .

Cytotoxicity assay of DC–enriched splenic leukocyte population on CC531 monolayer

DC-enriched splenic leukocytes population or PBM or LSWC were laid upon CC531 monolayer in E/T ratio 1:2. Alternatively, small pieces of spleen were situated on CC531 monolayer. Incubation time lasted 24, 48 and 72 hours. Bald spots in CC531 monolayer around spleen and other cells were counted under fluorescence reversed microscope.

In vitro adherence test of DC–enriched splenic leukocyte population, PBM and LSWC to liver metastases

Our previously described method was used (18). Rat liver specimens were obtained 4 to 6 weeks after inoculation of CC531 cells. Fragments of 5 x 5 x 5 mm were snap frozen in liquid nitrogen and stored at -70° C. Cryostat sections of 5 u were cut at -20° C and dried for 2 h. Then 100 microliters of suspension containing 1,5 x 106 of mononuclear cells were applied on sections and incubated under rotation for 30 min at 4° C. The non adhering cells were rinsed off with cold PBS. To determine expression of surface molecules of adherent cells staining with mAb anti: CD43, MHCII (OX6), OX62, CD14 (ED1) and CD161a (NK) was performed. Mononuclear cells which adhered to the sections stood as darkly stained cells above the plane of the tissue itself. Cells stained with monoclonal antibodies were recognized by their colored rim of membrane and cytoplasm. The number of cells adhering to liver tissue, tumor cells and matrix was counted using microscopic picture analyzer (Microimage Olympus, Japan).

Statistical evaluation

For statistical evaluation student t-test was applied with significance at <0.05.

Results

Phenotypical analysis of DC–enriched splenic leukocyte population

In the DC-enriched population of a normal spleen, 50% of isolated cells expressed class II, 25% CD14, 15% W3/13, 12% OX62, almost 70% light kappa chain (OX12), 30% showed B cell specific antigen and about 18% made up cells expressing 161a (NK). We

Table2

Phenotypical characteristics of DC-enriched splenic leukocyte population following in vivo 6-day stimulation with LPS, expressed in percent, *p<0,05

GROUPS	ANTIBODY OR SURFACE ANTIGEN						
	MHC classII	CD14	W3/13	OX62	OX12	B cells	CD161a
DC control	47,4± 16.0	26,2± 8,7	14,7± 8,7	12,2± 4,7	67,6± 14,6	29,5± 16,2	18,8± 12,4
DC + LPS	80,5± 10,5*	13,7± 3.0*	38,1± 5,3*	5,2± 6,9*	70,8± 13,7	22,3± 24,5	7,2± 5.0*

Table3

Densitometric data of Western Blot identification of proteins produced by DC-enriched splenic leukocyte population following 6-day in vivo stimulation with LPS (n=10), (density units), *p<0,05

PROTEIN	DC control	DC +LPS
TLR2	1627,5± 415,1	3920± 261,6*
TLR4	0	430,5± 137,9*
TLR9	0	0
HSP60	0	1353,5± 169*
HSP90	0	5049,5± 550,8*

concluded that specific markers for the DC population should be CD14, class II and OX62 antigens (Table 2). After 6 days in vivo stimulation with LPS, there was more cells expressing MHC class II antigen and W3/13+ (p<0,05), however, less of OX62+, CD14+, and CD161a+ (p<0,05) (Table 2). The CD4+25+ (Treg) cells were represented in 4± 1 percent to increase after immunization to 6± 2 percent (NS).

Western blot analysis of protein expression by DC-enriched splenic leukocytes population.

In the control population of DC-enriched splenocytes only TLR2 expression was observed. Stimulation with LPS caused strong expression of TLR2 and TLR 4, as well as HSP60 and 90 (Table. 3).

IL-12 and IFN-γ cytokines secretion by DC-enriched splenic leukocyte population

In vivo stimulation of DC-enriched splenic leukocytes population with LPS brought about decrease of IL12 production from 38 pg/ml in controls to 18 pg/ml. IFN-γ expression increased after stimulation of this population from 128,5 pg/ml to 387.5 pg/ml.

Rosette formation between CC531 and DC enriched splenic leukocytes population

Only sporadic rosette formation between CC531 and DC-enriched splenic leukocyte population was observed in non-immunized rats (Fig.1A). It was 3± 1 for CC531-DC, 3± 2 for PBM and 5± 2 for LSWC. No statistically significant differences were observed between the immunized/ LPS boosted and non-immunized animals. We did not observe any preponderance of adhering cells phenotype. The MHC class II, W3/13, CD14, OX62 and CD161a were evenly distributed in the microscope field and mostly apart from CC531 cells.

Cytotoxicity of DC-enriched splenic leukocyte population against CC531 cells

The cytotoxicity level against CC531 cells at E/T ratio 40:1 was

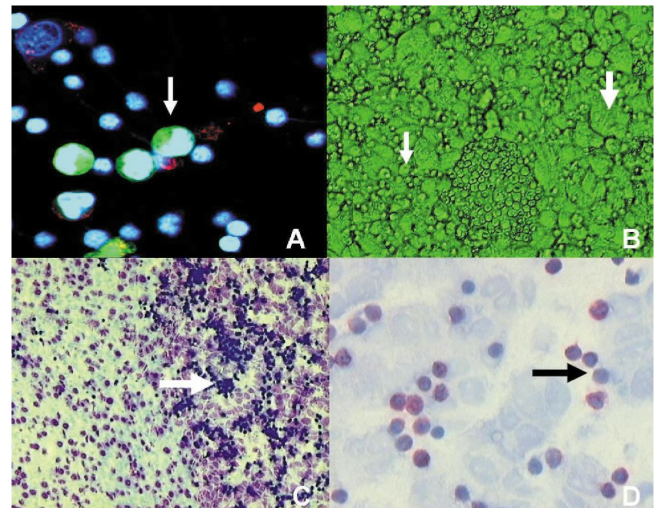

Fig. 1. A. Confocal microscopy picture of a 24h culture of CC531 cells (large, arrow) and splenic DC-enriched mononuclear cells (small). Only one small MHCII+ (OX6+) cell attached to CC531 cell, otherwise host cells loosely distributed in the field; B. Monolayer of CC531 cells overlaid with splenic DC-enriched cells in a 24h culture. No bold areas indicating lysis of tumor by spleen cells. Tumor cells- thick arrow, mononuclear cells- thin arrow; C. CC531 liver metastasis with overlaid splenic DC-enriched mononuclear cells evidently more attaching to the tumor (arrow) than liver tissue; D. CC531 liver metastasis with overlaid splenic DC-enriched mononuclear cells evidently more attaching to the tumor stroma (arrow) than tumor cells.

for DC-enriched splenic leukocyte population 15± 3 %, for PBM 4± 1% and for LSWC 17± 3 %. No statistical difference in level of cytotoxicity was found between CC531-immunized/ LPS boosted and non-immunized animals.

Cytotoxicity assay of DC-enriched splenic leukocyte population on CC531 monolayer

DC-enriched splenic leukocyte population, PBM or LSWC were laid upon CC531 monolayer in E/T ratio 1:2. No bald spots in CC531 monolayer around effector cells were observed (Fig. 1B).

Migration of splenocytes to the liver

FITC-stained splenocytes injected into the spleen flowed out

from that organ immediately following release of clamp from vascular peduncle. Only few stained cells remained in the parenchyma. On liver slides single stained cells were found in the sinusoids 1, 2 and 6 h after injection. Only sporadic FITC-stained cells were detected in the neoplastic tissue.

Test of adhesion of splenocytes, LSWC and PBM cells CC531 immunized rats to the colon cancer liver metastases in vitro

Spleen DC-enriched population, LSWC and PBM cells showed higher adherence capacity to the tumor than normal liver tissue (Fig. 1C) (Table 4). Interestingly, adherence rate was higher to tumor stroma than to tumor cells (Fig. 1D) (Table 5). With respect to the adhering phenotypes of DC-enriched population and LSWC , the MHCII and CD14 cells revealed higher tendency to adhere to tumor and adjacent liver tissue than CD161a and OX8 cells (Table 6). There were no differences in the adhesion rate of various phenotypes to tumor and liver sections. Neither, were there differences between control and immunized specimens.

Discussion

The fundamental principle permitting anti-tumor vaccination is the specific function of dendritic cells (DC). DC are the most potent antigen presenting cells, they are capable of inducing cell-mediated immunity through the promotion of Th1 responses and directly stimulate CTL. Moreover, cell-mediated immunity mediated by CTL seems to be more effective in eradicating cancer cells than any other cell type. Many investigators have hypothesized that DC can be used to immunize patients against their own cancers. However, anti-tumor vaccination contains a number of flaws, among them that only a few percent of committed cells reach the cancer focus. Therefore, it seems rational to create an in vivo model of vaccination with the specifically primed DC and cytotoxic T lymphocytes being continuously delivered to the liver vascular bed. This could be achieved by mobilizing anti-tumor immunized spleen migrating populations.

We immunized rats with CC531 tumor cells and stimulated them with E. coli LPS. Subsequently, spleen DC-enriched population was investigated for its anti-tumor cytotoxicity and adherence to tumor tissue. The migrating fraction of splenocytes flows to the liver via splenic vein and homes in liver sinusoids. They are called the liver sinusoidal wash-out cells (LSWC). We also isolated this population and checked for cytoxicity and adherence as it was done with splenic cells. The study provided the following information: a) immunization with CC531 cells and stimulation with LPS of splenic population containing DC, NK cells and lymphocytes activated these cells, but did not significantly increase the cytotoxicity against CC531 cells. This was seen both in the in vitro DC-CC531 cluster formation and cytotoxic tests. Adhesion of splenic DC to liver CC531 metastases on cryosections was higher than to the adjacent liver tissue. However, it was more expressed on tumor stromal than neoplastic cells. The level of splenic Treg cells down-regulating immune response was found only slightly increased after immunization. Taken together, low level of spleen cellular reactivity to tumor in the in vitro direct cluster formation between spleen effector and CC531 cells and cytotoxicity as well

Table 4
In vitro adhesion of PBM cells, spleen DC-enriched population and LSWC to CC531 metastases and normal liver tissue sections in control and immunized rats expressed in percent ± SEM, n=10

Cell type	Tumor surface Control Immunized	Normal liver surface
PBM	77 ± 3.5 80± 3.0*	22 ± 1.5**
Spleen DC-enriched	65 ± 3.0 70± 4.0*	40 ± 3.0***
Normal LSWC	62 ± 3.5 69± 8.0*	43 ± 3.5****

PBM– peripheral blood mononuclear cells, DC–dendritic cells, LSWC–liver sinusoidal wash–out cells. * control vs immunized non–significant, control and immunized vs normal liver surface ** p<0.05, ***p<0.005, ****p<0.0001

Table 5
In vitro adhesion of PBM cells, spleen DC-enriched population and LSWC to CC531 cells and tumor stroma sections in control and immunized rats, expressed in percent ± SEM, n=10

Cell type	Tumor stroma Control Immunized	Tumor cells Control Immune
PBM	80 ± 1.5 83± 6.0*	17 ± 1.5**15± 2.0***
Spleen DC-enriched	82 ± 1.5 79± 7.0*	18± 2.0**20± 3.0***
Normal LSWC	84 ± 1.7 82± 9.0*	16± 1.5**17± 6.0***

PBM– peripheral blood mononuclear cells, DC–dendritic cells, LSWC–liver sinusoidal wash–out cells. * control vs immune stroma non–significant, ** control tumor stroma vs control stroma p<0.0001, ***immune tumor stroma vs immune tumor, p<0.0001

Table 6

Phenotypes of cells in vitro adhering to tumor sections in controls and after immunization in percent (n=16)

Antigen Cell type	Cell population laid upon tissue sections	% of adhering cell phenotypes	
		Tumor Control	Tumor Immunized
MHCII (OX6)			
PBM	18± 1.9	20± 5.3*	22± 5.0*
Spleen DC- enriched	16± 2.3	45± 8.0**	42± 10.2*
LSWC	26± 8.6	40± 9.8***	43± 12.0*
CD14 (ED1)			
PBM	18± 2.5	50 ± 8.0****	49± 14.0*
Spleen DC -enriched	14± 4.0	25± 2.0***	18± 4.0*
LSWC	30± 8.0	23± 2.5***	30± 6.0*
CD161a (NK)			
PBM	8 ± 4.0	18± 10,0***	17± 4.0*
Spleen DC- enriched	30± 15.0	25± 3.0*	29± 10.0*
LSWC	26± 4.0	21± 10.0*	22± 10.0*
OX8(CD8)			
PBM	25 ± 3.0	32± 3.0***	34± 10.0*
Spleen DC- enriched	50± 5.0	50± 6.0*	41± 9.0*
LSWC	53± 2.0	49± 1.0*	56± 13.0*

Cells laid upon liver sections vs adhering to tumor * NS **p<0.005 ***p<0.01 **** p<0.001,

as low adherence rate to tumor cells were observed despite of anti-tumor immunization with LPS boosting.

The first problem we encountered in our study was isolation and characterization of rat spleen DC. Human DC express characteristic cellular markers, including substantially higher levels of antigen-presentating molecules(e.g., CD1a, MHC-I, and MHC-II) than any other cell in the body (19). In addition, DCs have a high surface density of accessory/ costimulatory molecules, including leukocyte functional antigen (LFA)-3/CD58, B7-1/ CD80, B7-2/CD86, CD40, and intracellular adhesion molecules (20). Since there is no specific marker for rat splenic DC we decided to use a DC-enriched population, obtained by a gradient isolation. This population contained large cells of class II (OX6) and CD14 (ED1) phenotypes and admixture of small CD161a (NK),CD8 (OX8) and B cells. These cells cooperate in the spleen in reaction to bacterial and presumably tumor antigens.

DCs residing in tissues are in an "immature" state (iDC) and are unable to stimulate T cells. In vivo, DC maturation occurring in response to the microen vironmental signals allows DC to switch their functional phenotype from antigen-processing cells when immature to T-cell stimulatory cells when mature. Fresh spleen DC are poor stimulators of CD4+ and CD8+ T cells, but become potent inducers of T cell proliferation as well as Th1 differentiation after stimulation by CpG (15). To obtain mature DC we stimulated splenocytes by in vivo administration of E. coli LPS and this effect has been well documented by high expression of TLR, Hsp 60and 90 and increased production of INFγ. In contrast, immunization by in vivo administration of CC531 cells or their debris could not be easily proved as the humoral response to tumor antigen was very low.

The cellular response to immunization is mediated by DC ingesting tumor apoptotic bodies, processing and presenting to T lymphocytes(14, 21). Specific cytotoxicity of CTL proves efficacy of the immunization protocol. We did not observe any significant increase in the DC-enriched population cytotoxicity against immunizing CC531 cells above the control pre-immunization level seen in the splenic and LSWC populations. The DC cooperate with T and NK cells. It is known that tumor antigen-pulsed DC effectively suppress the growth of hepatocellular carcinoma in mice. By inducing DC activation, NK-cell activation induced by tumor cells can indirectly promote antitumoral T-cell responses. Reciprocally, DC activated through Toll-like receptors (TLR) induce potent NK-cell activation in antiviral response (22-25).Our studies showed that the splenic DC-enriched population contains high percentage of CD161a (NK) and OX8 cells, largely exceeding the PBM values. This population revealed higher cytotoxic activity and in vitro adherence to tumor sections than the PBM population. Interestingly, the splenic population did not differ in its activity with LSWC. Immunization with CC531 did not enhance this effect.

The observed lack of effect of immunization and LPS boosting on antitumor activity observed by us has been has been reported by others. It has been shown that tumor infiltrating lymphocytes are

compromised and contain a subpopulation of suppressive CD4+CD25+Foxp3 Treg cells. Local immune responses in patients with cholangioma are held in check by tumor infiltrating CD4+CD25+ T-regulatory lymphocytes, which suppress the activity and proliferation of effector CD4+ and CD8+ T cells. Treg cells isolated from HCC suppressed proliferation of autologous circulating CD4+CD25+ cells and perforin expression and proliferation of autologous CD8+ T cells (26, 27, 28). We found only 4% of CD4+25+ in the splenic and LSWC populations. There was no significant increase in frequency of this population after immunization with CC531, neither did boosting with LPS do so. We did not search for myeloid suppressor cells in this report.

Another explanation of the low effectiveness of immunization may be the observed defectiveness of DC. Defective DC function in cancer patients is the result of decreased numbers of competent DC and the accumulation of immature cells. The major finding is the lack of expression of co-stimulatory molecules in tumor-associated DCs consistent with the phenotype of immature, nonactivated DC. A population of DC isolated from the peripheral blood of patients with breast and head and neck cancer demonstrated significantly reduced ability to cluster and stimulate allogeneic and antigen-specific T cell responses (29, 30).

In our previous studies on the immunological cellular reactions against CC531 tumor line we showed that population of mononuclear cells washed-out from the liver sinusoids reveals diminished cytotoxic activity against CC531 and K562 tumor lines (31). Low level of LSWC cytotoxicity against CC531 indicates that sinusoidal cells lose their cytotoxic potential in the presence of tumor cells.

A frequently raised question is about migration of primed DC and CTL to the tumor site. It has been shown that migration of ex vivo generated DC is rather inefficient. When injected i.v. these cells localize to the lung before being visible in the spleen and liver. Binding of NK cells to liver neoplastic tissues was observed only after i.a. injections (32-35). We tried to overcome this problem by in vivo stimulating splenic cells and use spleen as a physiological source of DC and CTL released to the splenic vein and subsequently homing in liver. The FITC-labelled cells located in liver sinusoids, although in low numbers. We did not so far study the specific trapping of labeled cells in liver tumor tissue. Another test to prove predilection of DC to tumor cells, applied in this study, was based on the adherence properties of immune cells to the inflammatory foci. In our previous studies we showed that specific halting of MHCII+CD14+, CD8+ and CD11c+ leukocyte subpopulations takes place in the ex vivo perfused liver bearing CC531 metastases and that these cells adhere to the frozen sections of CC531 more frequently than to the normal liver tissue. This test was repeated in the present study and showed adhesion properties of DC-enriched population, LSWC and PBM cells to the metastatic foci, however, mosty to the stroma but not neoplastic cells. Surprisingly, there were no specific cell phenotypes which attached to tumor cells compared with normal liver tissue. Moreover, no differences in phenotypes were seen in the adherence rate to tumor and adjacent liver tissue. Furthermore, no differences were observed between the immunized and non-immunized animals. This last observation may be accounted for by presumptive lack of tumor-specific adhesion molecules on CC531 cells.

The role of spleen cells in elimination tumor antigen or enhancement of tumor growth remains controversial. It has been suggested that whether splenectomy enhances or inhibits tumor growth depends primarily upon the ratio of spleen to tumor. Isolated splenic metastasis is a rare finding in the follow-up of colorectal cancer patients and long-term survival can be achieved with splenectomy (36-40). This creates suggestions that spleen cellular environment is active in eliminating metastatic tumor cells. Is this the effect of cooperation of various resident and migrating populations in their spatial context, remains to be clarified. Only migrating cells are physiologically released into the splenic tein. Probably without other supporting cells they are ineffective in attacking tumor cells. They home to the liver and participate there in the tolerizing processes. Is the tolerizing property of liver of advantage for the tumor or host requires elaboration (41, 42).

Taken together, low level of spleen cellular reactivity to tumor in the in vitro direct cluster formation between spleen effector and CC531 cells and cytotoxicity as well as adherence to liver tumor metastases were observed despite of anti-tumor immunization with LPS boosting.

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