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Research Paper

Primary tumor cells obtained from MNU-induced mammary carcinomas show immune heterogeneity which can be modulated by low-efficiency transfection of *CD40L* gene

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Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; CD40L, CD40 ligand; RMECs, rat mammary epithelial cells; RMTCs, rat mammary tumor cells; pCD40L-t-RMTCs, RMTCs transfected with recombinant CD40L; pIRES2-t-RMTCs, RMTCs transfected with empty vector; pCD40L-t-NMU, NMU cell line transfected with recombinant CD40L; pIRES2-t-NMU, NMU cell line transfected with empty vector; MLTR, mixed leukocyte-tumor reaction

Key words: immune heterogeneity, primary cell culture, mammary cancer, transfection, CD40, CD40 ligand

The presence of CD40 on carcinoma cells is an important factor for the generation of tumor-specific responses induced by CD40 ligation. In an *N*-methyl-*N*-nitrosourea (MNU)-induced autochthonous mammary tumor model, we analyzed the immune features of primary tumor cells. Here, CD40 was frequently detected on the primary tumor cultures and selectively expressed on the malignant mammary tissue in vivo. On the other hand, every mammary tumor cell culture had a heterogeneous and reduced expression of proinflammatory *TNFα, IL-1β, IL-6* and *CXCL1* cytokines compared to normal mammary epithelial cells. Low-efficiency transfection of *CD40 ligand (CD40L)* gene enhanced the expression of proinflammatory cytokines in the tumor cells and strengthened allogeneic immune reactions and costimulatory activity which may help overwhelming suppressive features of the tumor.

Introduction

Breast cancer is the most frequently diagnosed cancer in women while high grade tumors exhibit a certain heterogeneity facilitating metastatic spread, drug resistance and relapse during conventional cancer therapies.[1,2] The understanding of interactions between cancer cells and immune system may lead to the invention of novel therapeutics which enhance the anti-tumor immune response. However, suppressive and heterogeneous nature of the tumor cells diverts the immune system to respond differentially at individual level.[2]

Autochthonous tumor models may serve to study heterogeneous populations of neoplastic cells and chemical carcinogenesis is utilized conventionally to establish autochthonous tumors in laboratory animals.[3] Mammary tumor models induced with MNU in Sprague-Dawley rats have long been regarded as being relevant to human mammary carcinogenesis due to the fact that the tumors carry a similar histopathology with human breast lesions.[4,5] Thus, this experimental tumor model has been widely used for chemoprevention and carcinogenesis studies.[6,7]

CD40, a member of the tumor necrosis factor receptor superfamily, is commonly expressed on antigen presenting cells. Engagement of CD40 with its ligand, CD40L, enhances the antigen presentation and the expression of other costimulatory molecules, such as CD80 and CD86 resulting in an indirect crucial role for CD40L in T-cell priming.[8,9] CD40 may also be found on transformed cells.[10] Besides hematological malignancies (except T-cell lymphoma/leukemia), CD40 can be observed on solid tumors, including a portion of the human breast cancers.[10-13] Stimulation of CD40 modulates the expression of several molecules on carcinoma cell lines including several surface molecules and cytokines.[10,14] The data obtained from the tumor models established with transplantable cell lines in syngeneic inbred animals evidenced that genetic-modification of $CD40^+$ or $CD40^-$ tumor cells with CD40L can result in protection and effective systemic anti-tumor immune responses.[14-16]

Here, we investigated the CD40-related immune features of primary mammary tumor cells obtained from chemically-induced autochthonous rat mammary tumors. The aim of the current study was to validate a possible immunotherapeutic application via CD40

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molecule for heterogeneous mammary tumors, ex vivo. Therefore, a comparative analysis of proinflammatory cytokine expression was performed between primary rat mammary epithelial cells (RMECs) and rat mammary tumor cells (RMTCs). Besides, it was determined whether liposomal transfection of *CD40L* gene into the primary mammary tumor cells can modulate the cytokine expression and immune reactions.

Results

CD40 expression on MNU-induced mammary tumors. Following the injections of MNU, a total of 18 mammary tumors was developed in the Sprague-Dawley rats. Three of the tumors were diagnosed as epithelial hyperplasia whereas the rest was histopathologically classified as ductal carcinoma. These malignant tumors displayed several combinations of papillary, cribriform, solid and/or comedo patterns. There was no correlation amongst tumor localization, diameter and histopathology (data not shown).

CD40 expression on the MNU-induced mammary tumors was studied by immunohistochemistry. A rare and slight staining for CD40 was observed on the epithelial hyperplasia. In 14 out of 15 cases of ductal carcinoma, a diffuse staining was seen with high or moderate intensity (Fig. 1A and B). The mammary epithelium in the tumor-free regions of the rats administered with MNU was negative for CD40 (Fig. 1C). On the other hand, in the mammary tissue areas adjacent to the malignant tumor, a few epithelial cells were positive for CD40 (Fig. 1B). CD40 was also detected on stromal components of the mammary tissue such as infiltrating histiocytes, endothelial cells and myoepithelial cells.

CD40 expression on RMECs and RMTCs, in vitro. We established six primary RMEC and six primary RMTC cultures each isolated from a different outbred rat. NMU cells were used as cell line counterpart of the primary mammary tumor cells. CD40 was expressed on 15.9 ± 1.4% of the RMECs and on 26.1 ± 3.9% of the RMTCs. On the other hand, NMU cells were highly positive (69 ± 2.1%) for CD40 with a low-level surface expression (Fig. 2A–C). Also, CD40 gene expression was higher in the RMTC cultures and the NMU cell line compared to the RMECs (Fig. 2D). The presence of CD40 on the primary cells was also confirmed by direct immunofluorescence staining (Fig. 2E).

Expression of proinflammatory cytokines in the primary cell cultures. TNFα, IL-1β, IL-6 and CXCL1 play a potent role in the activation and recruitment of immune cells into malignant lesions.[17] We detected *TNFα, IL-1β, IL-6* and *CXCL1* gene expression both in the normal epithelial and tumor cultures. However, these cytokines were expressed at lower levels in the RMTCs (Fig. 3). The average $2^{-\Delta\Delta Ct}$ value indicated a similar *IL-6* expression between the RMECs and RMTCs. Among the RMTC cultures, only two expressed *TNFα* and three expressed *IL-6* slightly higher than the normal epithelial cells. On the other hand, *IL-1β* and *CXCL1* expression were relatively high in NMU cells.

Effect of pCD40L transfection on the *TNFα, IL-1β, IL-6* and *CXCL1* gene expression. Stable gene transfer into primary cells is a challenging approach.[18] Correspondingly, the RMTCs could only be transiently transfected whereas stable transfected NMU cells were obtained after neomycin selection. The efficiency of transient-transfection in the RMTCs was 12.2 ± 2.9% (Fig. 4A). In accordance with EGFP expression, de novo expression of *CD40L*

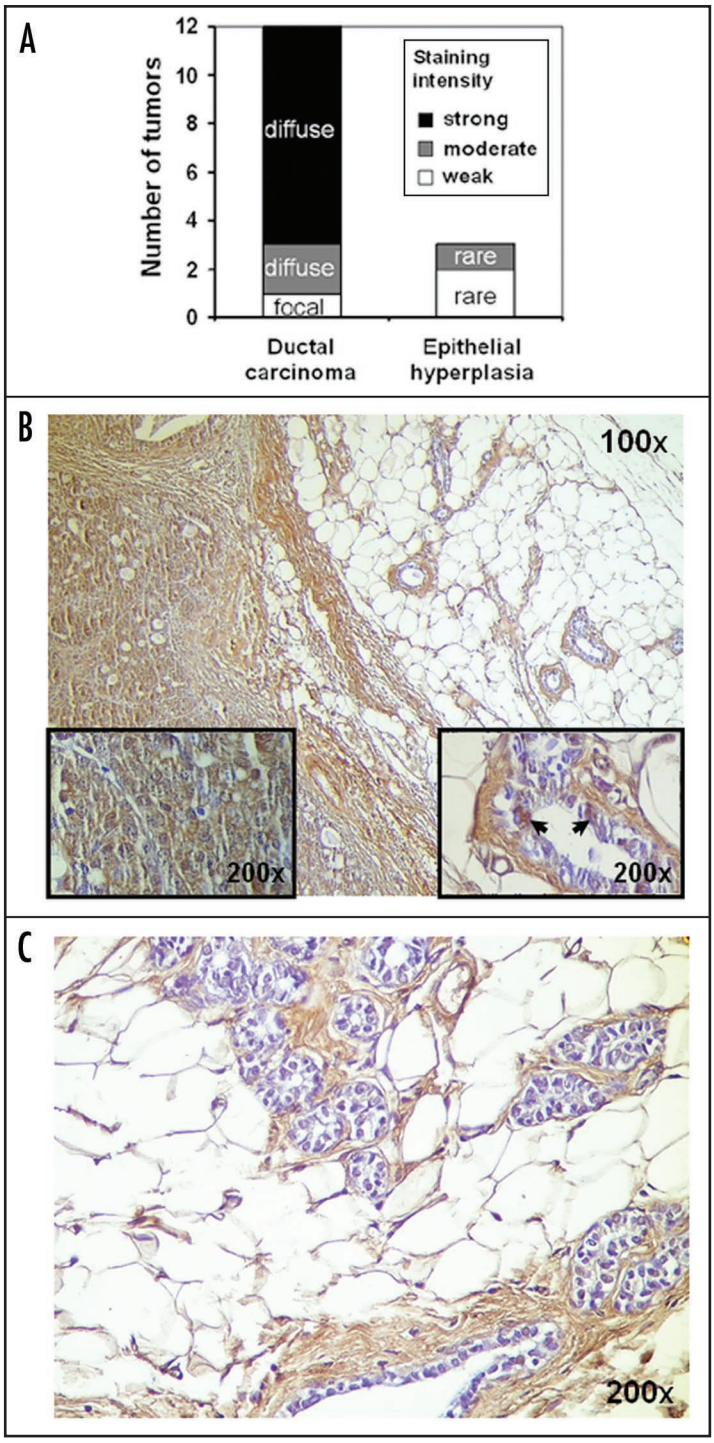

Figure 1. Immunohistochemical analysis of CD40 on MNU-induced mammary tumors and on normal mammary tissues. (A) CD40 expression on mammary tumors was evaluated according to staining pattern and intensity. In (B) diffuse pattern of a CD40 staining in a representative ductal carcinoma is shown. Higher magnifications of the tumor tissue and the non-malignant ductal epithelium (arrow heads show the $CD40^+$ epithelial cells) residing next to the tumor can be seen at left- and right-hand corners, respectively. (C) CD40 staining was absent on the mammary epithelium in the tumor-free regions. CD40 positivity can be seen on the stromal components and myoepithelium.

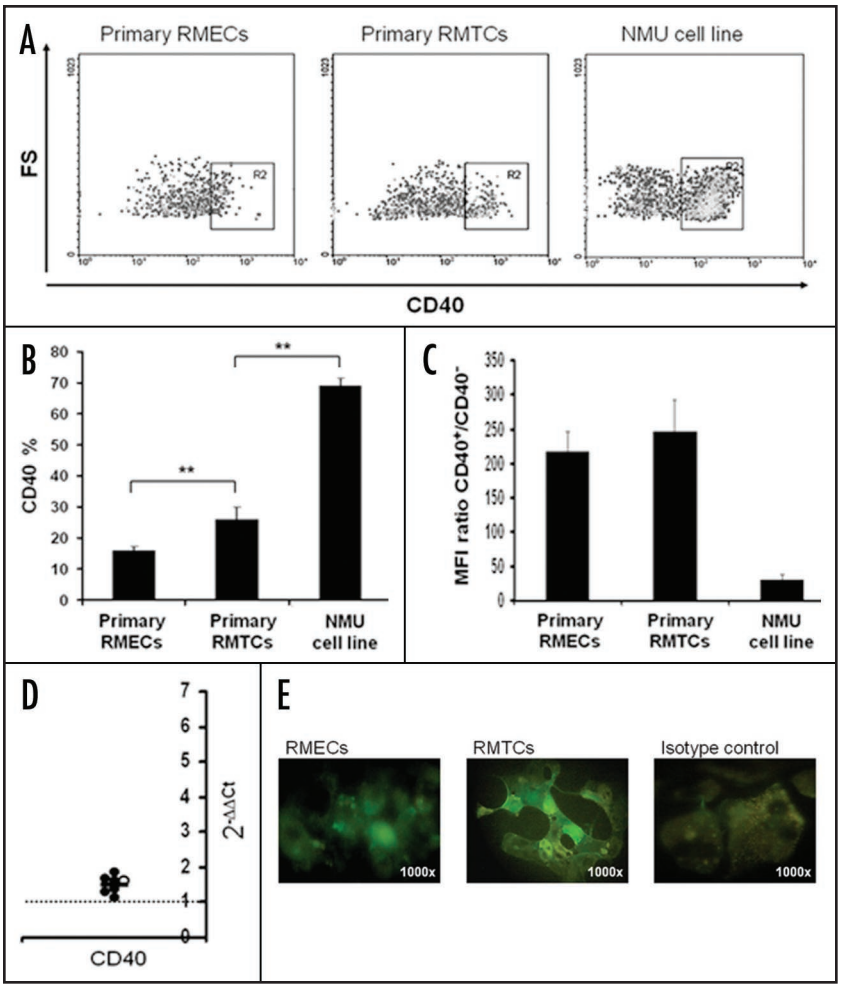

Figure 2. Expression of CD40 in the primary cell cultures. CD40 expression was studied by flow cytometry. (A) Dot-plots of representative cultures of primary RMECs, RMTCs, and NMU cell line are shown. CD40+ cells are demarcated in the region R2. (FS, forward scatter). The percentage of CD40+ cells (B) and its surface expression level (C) were determined in RMECs (n = 4), RMTCs (n = 4) and NMU cell line (**p < 0.01). (D) *CD40* gene expression was determined by real-time RT-PCR. The data obtained from RMTCs or NMU cells were normalized against RMECs (closed circles, RMTC cultures; open circles, NMU cell line). Dashed line crossing at the value $2^{-\Delta\Delta Ct} = 1$ indicates an equal *CD40* expression level between RMECs and RMTCs or NMU cells. The average value of the RMTC gene expression has been indicated with a short line. (E) The immunofluorescence micrographs of CD40+ cells in the representative primary RMEC and RMTC cultures. Autofluorescence and isotype control staining of the cells can also be seen at the right-hand panel.

gene was also determined in the pCD40L transfected RMTCs (pCD40L-t-RMTCs) while the cells transfected with the empty vector (pIRES2-t-RMTCs) were negative (Fig. 4B).

The effect of pCD40L transfection on the proinflammatory cytokines expressed in RMTCs was determined in comparison with the empty vector transfections. *TNFα, IL-1β* and *IL-6* was increased in the pCD40L-t-RMTCs (Fig. 4C). *TNFα* had the most diverse expression and two of the pCD40L-t-RMTC cultures were negative for *TNFα*. Only one pCD40L-t-RMTC culture did not express *IL-6. CXCL1* expression was not modulated in pCD40L or pIRES2 transfections. The data obtained from the NMU cell line transfected with pCD40L was correlated with the pCD40L-t-RMTC data however *TNFα* expression was not increased in the NMU cells.

Allogeneic immune reactions were enhanced with pCD40L-t-RMTCs. Interaction between the primary tumor cells and allogeneic immune cells was investigated to reveal potential effects of the low-efficiency pCD40L transfection on immune reactions, ex vivo.[19] Three independent mixed leukocyte-tumor reactions (MLTRs) was performed with different RMTC cultures transfected with pCD40L or pIRES2 and allogeneic splenocytes obtained from several outbred rats. Allogeneic splenocyte proliferation was significantly increased with the RMTCs and NMU cells transfected with pCD40L at 72 hours (Fig. 5A and B). Interestingly, there was no difference between pIRES2-t-RMTC MLTRs and control splenocytes cultured alone (Fig. 5A).

Since the allogeneic immune reactions with pIRES2-t-RMTCs were unexpectedly deprived, next, we investigated the expression of costimulatory molecules in MLTRs (Fig. 5C and D). Suppression or impediment on CD80 and CD86 was relatively consistent at 72 and 96 hours, however in some reactions; allogeneic recognition was expectedly potent enough to induce immune activation by the time.

In the experiments performed with the transfected NMU cell line, especially, a decline in CD86 expression was evident in the pIRES2-t-NMU MLTRs. On the other hand, as observed in the MLTRs with RMTCs, the NMU cells carrying CD40L were able to enhance the CD80 and CD86 expression.

Discussion

In a chemically-induced autochthonous mammary tumor model, we examined the CD40-related immune features of the primary tumor cells. Primary tumor cultures can be more representative for the heterogeneity of patient-derived tumor samples than the cell lines.[20] In this study, CD40 was more frequently detected on the primary tumor cells than on the normal mammary epithelial cells, ex vivo. Moreover, CD40 was selectively expressed on the malignant mammary tissue, in vivo. On the other hand, every primary tumor culture had a divergent and reduced expression of proinflammatory genes compared to normal mammary epithelial cells. Upon introduction of *CD40L* gene into the primary tumor cells, the expression of proinflammatory cytokines was enhanced and the immune reactions were strengthened, ex vivo.

Consistent with our observations on the MNU-induced rat mammary tumors, CD40 is also expressed on a portion of human breast cancers but it is rarely found on histopathologically normal mammary epithelia.[11,12,21] The low-level expression of CD40 on hyperplasia may be attributed to the extent of local stress. It is well evidenced that CD40 can be induced on the epithelial tissue under stress conditions such as inflammation, harsh milieu of tumor or in vitro conditions.[13]

The persistent expression of CD40 must be regulated by local mediators present in the tumor microenvironment. Since, even in the RMTCs isolated from the tumors diffusely expressing CD40,

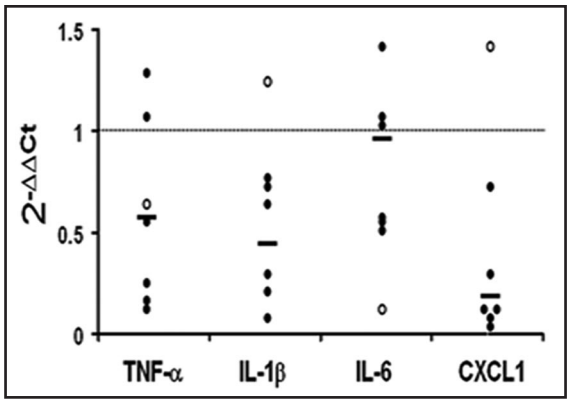

Figure 3. Expression of *TNFα, IL-1β, IL-6* and *CXCL1* genes in primary RMTCs and NMU cell line. The real-time RT-PCR data obtained from RMTCs or NMU cells were normalized against RMECs (closed circles, RMTC cultures; open circles, NMU cell line). Dashed line crossing at the value $2^{-\Delta\Delta Ct}$ = 1 indicates an equal expression level between RMECs and RMTCs or NMU cells for the relevant gene. The average value of the RMTC gene expression has been indicated with a short line.

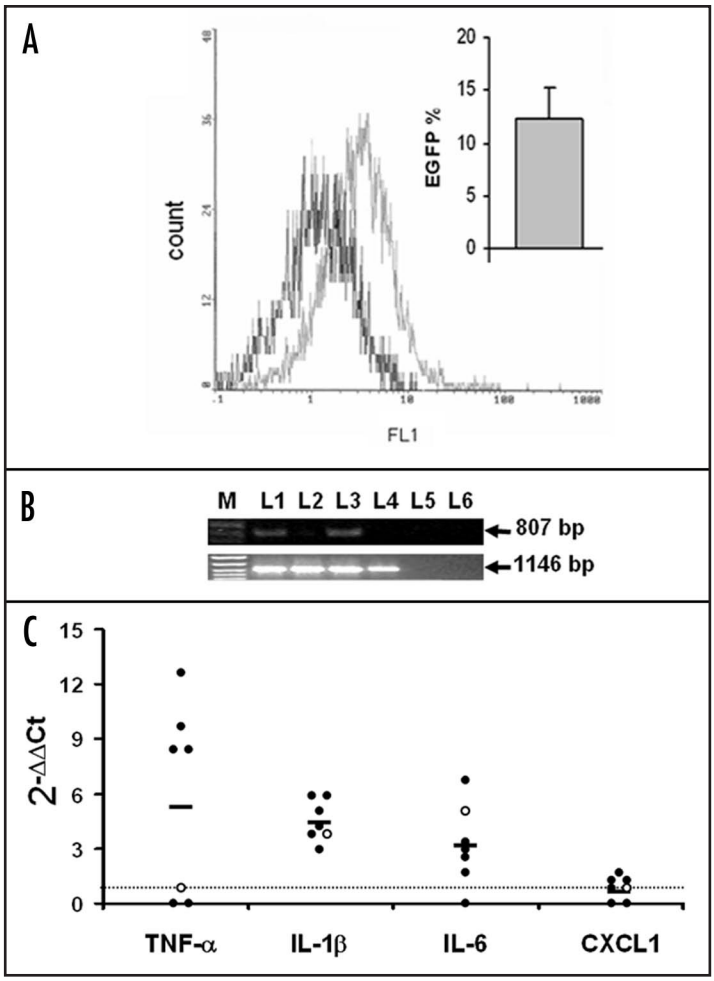

Figure 4. Modulated expression of proinflammatory cytokines in RMTCs or NMU cells transfected with pCD40L. (A) Liposomal transfection efficiency in primary RMTC cultures at 48 hours post-transfection was evaluated by flow cytometry. EGFP expression in a representative RMTC culture transfected with pCD40L is shown (n = 3). (B) Expression of *CD40L* in representative RMTC cultures transfected with pCD40L (pCD40L-t-RMTCs) or empty vector (pIRES2-t-RMTCs). Upper panel shows CD40L and lower panel shows *β-actin* RT-PCR results. M, 100 bp DNA ladder; L1, L3, pCD40L-t-RMTCs; L2, L4, pIRES2-t-RMTCs; L5, RT-negative PCR control of pCD40L-t-RMTCs; L6, PCR negative control. (C) *TNFα, IL-1β, IL-6* and *CXCL1* gene expression was analyzed by real-time RT-PCR, (closed circles, pCD40L-t-RMTCs cultures; open circles, pCD40L-t-NMU). The data from pCD40L-t-RMTCs or pCD40L-t-NMU were normalized against empty vector transfected pIRES2-t-RMTCs or pCD40L-t-NMU, respectively. Dashed line crossing at the value $2^{-\Delta\Delta Ct}$ = 1 indicates an equal expression level of the relevant gene in the cells transfected with pIRES2 or pCD40L. The average value of the pCD40L-t-RMTC gene expression has been indicated with a short line.

the proportion of CD40$^+$ cells was reduced in culture condition. Furthermore, CD40 was induced on the RMECs ex vivo and its expression level was always lower than the RMTCs. In different RMTC cultures, *CD40* was rather homogeneous at the transcriptional level. To this end, our findings showed a selective and a homogenous expression for CD40, respectively in vivo and in vitro, in the MNU-induced mammary tumors obtained from outbred rats.

There are several studies addressing the CD40 pathway in the growth and differentiation of epithelial cells. CD40-mediated responses may also modulate the expression of several immune mediators in epithelial cells and regulate the local inflammatory reactions.[12] The cytokines, TNFα, IL-1β, IL-6 and CXCL1, examined in our study are previously reported to be commonly expressed by epithelial cells of several tissues including the mammary epithelium.[22-24] These proinflammatory cytokines serve as first signals to alert the immune system and to mediate inflammation in case of infectious or pathological circumstances.[25,26] Especially, TNFα and IL-1β have unfavorable effects on malignant mammary cells.[23,24] Hence, the decreased expression of these proinflammatory mediators in most of the primary RMTC cultures and in the NMU cell line in comparison to the normal mammary epithelial cells may serve as an evasion mechanism, hindering innate immunity thereby blocking further immune activation.

Interestingly, even though each RMTC culture was established from a mammary tumor induced by the same chemical agent, they exhibited certain heterogeneity in the cytokine expression levels. Here, we took the advantage of the primary RMTC cultures to study the possible outcomes of tumor heterogeneity on the immune responses, ex vivo.

CD40 ligation on several carcinoma cell lines was previously reported to enhance cytokine expression. For human mammary carcinoma cell line T47D, Alexandroff et al. reported an increase in IL-8 and CXCL1 (Gro-α) expression but they did not observe a change in IL-6 and TNFα upon stimulation with anti-CD40 antibody.[27] In our study, low-efficiency transfection of the primary RMTCs with CD40L enhanced the expression of *TNFα, IL-1β* and *IL-6* proinflammatory cytokines. Expectedly, RMTC cultures transfected with *CD40L* gene displayed a differential augmentation of the cytokine expression that again can be attributed to the heterogeneity of the cells. To this end, de novo expression of *CD40L* in the primary breast cancer cells can be plausible for anti-tumor immune reactivation. The enhancement of innate mediators may complement with adoptive immunity as the low-level expression of CD40L was previously shown to accelerate T-cell dependent responses.[28]

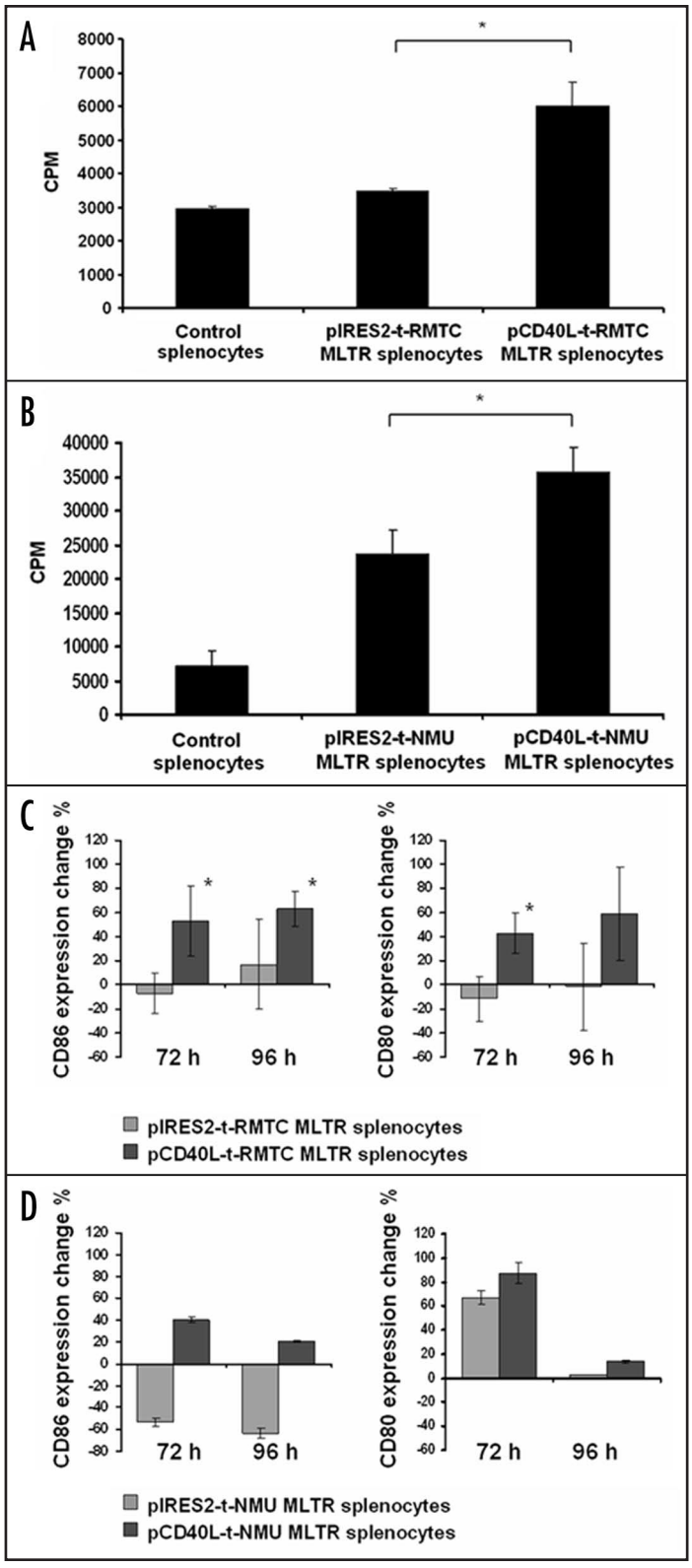

Figure 5. Allogeneic immune response in MLTRs. Splenocyte proliferation rates in response to allogeneic pCD40L-t-RMTCs or pIRES2-t-RMTCs (A) and to pCD40L-t-NMU or pIRES2-t-NMU (B) were analyzed by [^{3}H]-thymidine incorporation. Splenocytes cultured alone were used as controls for spontaneous proliferation. (C and D) CD80 and CD86 expression were determined by flow cytometry. The results obtained at 72 and 96 hours were compared with 48 hours data. Changes in the expression are shown in percentages (n = 3, *p < 0.05).

Under physiological conditions, allogeneicity causes a high effector cell proliferation in which the surface molecules CD80 and CD86 form the main costimulatory axis.[29] The alloantigen-specific response of the effector splenocytes was alleviated in the MLTRs with the sham transfected RMTCs. The immunosuppression of allogeneic immune reactions was also described by Banat et al. as a similar phenomenon observed with renal cell carcinoma cells.[19] However, in our study, the ex vivo immune responses were pronounced with the cells transfected with *CD40L*. Collectively, our data may demonstrate that the immunosuppressive behavior of the primary mammary tumor cells can be overwhelmed even by a scant amount of CD40L stimulation.

CD80 and CD86 have been reported to have differential effects on the immune costimulation.[30,31] The relative contribution of CD80 and CD86 to the overall data obtained from immune reactions with pCD40L-transfected tumor cells indicated that the costimulatory axis may be primarily sustained through the upregulation of CD86 expression.

Tumor models established with transplantable cell lines in syngeneic inbred animals are undoubtedly precise for conducting research on the biology of breast cancer. However, patient-derived mammary tumor samples display heterogeneity.[2,3,20] Here, in an ex vivo approach using an experimental mammary tumor model which is relevant to the human mammary carcinogenesis, we showed that the low-efficiency transfer of *CD40L* into primary mammary tumor cells can be useful for the enhancement and sustenance of immune reactions. Further in vivo studies are planned to investigate the efficacy of *CD40L* gene transfer for the mammary tumors heterogeneous in immune aspects.

Material and Methods

Animals and cell lines. Twenty outbred, 21-day-old female Sprague-Dawley rats were obtained from the Experimental Animals Breeding Unit of Hacettepe University. Ten rats were used for the chemical carcinogenesis experiments and the rest was used for normal mammary tissue controls. All rats were housed under environmentally controlled standard conditions. The Guiding Principles in the Care and Use of Laboratory Animals together with those described in the Declaration of Helsinki were strictly adhered in the conduct of all the experimental procedures described within this manuscript. This project was approved by the Institutional Animal Care and Use Committee of Hacettepe University, Ankara, Turkey (Approval Number: 2004/34-5) before its commencement.

The epithelial cell line NMU was previously established from a MNU-induced rat mammary adenocarcinoma.[32] It was obtained from the American Type Culture Collection (LGC Promochem, Rockville, MD, USA). NMU cells were cultured in Eagle's minimum essential medium (PAA, Pasching, Austria) containing L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). The cell culture supplements were purchased from Sigma (St. Louis, MO, USA).

Induction of mammary tumors and histopathological evaluation. *N*-Methyl-*N*-nitrosourea (MNU) (Sigma) was dissolved in physiological saline (pH 4.0) and injected intraperitoneally at a dose of 50 mg/kg when the rats were 21, 28, 35 and 42 days old. Mammary tumors with ~1 cm diameter were used in further experiments. The tumors were obtained from two independent

Table 1 **Primer sequences designed for quantitative RT-PCR**

Primer	Sense (5'–3')	Antisense (5'–3')	Product size	Accession[a]
CD40	GCTGCTTGTTGACAGCGGTCCA	AACTAGTCGGTTTCCCGGCTG	110 bp	NM_134360
IL-1β	CAAGCCCTTGACTTGGGCTGT	GGCAAGACATAGGTAGCTGCCAC	136 bp	M98820
IL-6	TCTCCGCAAGAGACTTCCAGC	ACTGGTCTGTTGTGGGTGGT	124 bp	M26744
TNFα	ACACATCTCCCTCCGGAAAGGA	TGAGAAGAGGCTGAGGCACAG	137 bp	NM_012675
CXCL1	AGCTCCAGCACTCCAGACTCCA	TGACAGCGCAGCTCATTGGCGA	144 bp	NM_030845
β-actin	ACCAGGGTGTGATGGTGGGTATG	CAGTTGGTGACAATGCCGTGTTC	118 bp	NM_031144

[a]NCBI nucleotide database accession numbers of the cDNAs available online at http://www.ncbi.nlm.nih.gov

chemical carcinogenesis experiments each performed with a group of five animals.

The tumors were excised, fixed in 10% formalin and then embedded in paraffin. Histopathological evaluation of the paraffin sections was done under conventional light microscopy after haematoxylin-eosin staining according to Russo et al.[4] and Singh et al.[5]

Immunohistochemical analysis of CD40. Immunohistochemistry was performed on 4 μm thick tissue sections using primary antibody against CD40 (dilution 1/50; 2 h incubation at 36°C) (clone C-20, Santa Cruz, CA, USA). Binding of the primary antibodies was detected with polyvalent secondary antibodies using a biotin/streptavidin/horseradish peroxidase detection system according to the manufacturer's protocol (UltraVision Polyvalent HRP-DAB chromogen; Lab Vision, Fremont, CA, USA). Antibody binding was visualized by using diaminobenzidine (DAB) tetrahydrochloride substrate. Immunostaining was evaluated by intensity of staining (weak, moderate, strong) and by staining pattern (<5%, rare; 5–50%, focal; >50%, diffuse) in comparison with isotype-matched (normal rabbit IgG, Santa Cruz) antibody controls.

Establishment and characterization of primary rat mammary epithelial cell (RMEC) and tumor cell (RMTC) cultures. Normal mammary fat pads or tumors were excised under sterile conditions and minced prior to incubation in DME medium containing 0.075% collagenase type II and 0.01% DNaseI (2 hours at 37°C). Suspensions of the digested tissue were strained and the primary cells were cultured in DMEM supplemented with β-estradiol (5 ng/ml), progesterone (0.5 μg/ml), hydrocortisone (0.4 μg/ml), epidermal growth factor (10 ng/ml), insulin (5 μg/ml), 10% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml). All reagents were obtained from Sigma. Fibroblasts were removed by selective-sequential trypsinization. Purity and epithelial origin of the cells were characterized by immunocytochemical staining of Muc-1 (epithelial mucin) glycoprotein (Muc-1 Ab-5, clone MH1; NeoMarkers, Fremont, CA, USA).[33] The primary cultures containing more than 96% Muc-1 positive cells at the third week of the first passage were used in the experiments. Additionally, the absence of adherent immune cells in the cultures is confirmed by absence of class II MHC (*RT1-Ba gene*) expression in RT-PCR analyses using sense, 5'-AGA GCT CTG ATT TTG GGG GTC and anti-sense, 5'-GCT GGA ATC TCA GGT TCC CAG TG primers.

Liposomal transfection and expression of recombinant rat *CD40L* gene. The complete coding sequence of the rat CD40L gene was previously cloned into pIRES2-EGFP eukaryotic expression vector (Clontech, Palo Alto, CA, USA) and the resulting recombinant construct was designated as pCD40L.[34]

Transfection of the plasmid DNA into primary RMTCs and NMU cells were optimized using (DNA-liposome ratio 1:2.5, five hours incubation) Lipofectamine2000^{TM} reagent (Invitrogen, Carlsbad, CA, USA) in DMEM. Stable transfected NMU cells were selected with antibiotic G418 (400 μg/ml). Transfection efficiency was determined by flow cytometry analysis of enhanced green fluorescent protein (EGFP) expression at 48 hours post-transfection. Untransfected cells were used for autofluorescence correction.

Expression of the recombinant *CD40L* was also tested by RT-PCR after the removal of plasmid DNA from RNA samples by rigorous DNase treatment (DNA-free; Ambion, Austin, TX, USA).[34] RT-PCR controls without reverse transcriptase (RT-negative PCR control) were performed to ensure the absence of contaminating plasmid or genomic DNA. PCR products were separated on 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

Mixed leukocyte-tumor reactions (MLTRs) and proliferation assay. The primary RMTCs transiently transfected with recombinant CD40L (pCD40L-t-RMTCs) or empty vector (pIRES2-t-RMTCs), or the NMU cells stably transfected with recombinant *CD40L* (pCD40L-t-NMU) or empty vector (pIRES2-t-NMU) were cocultured (48 hours post-transfection) with freshly isolated splenocytes from outbred rats, at 1:18 target-effector ratio for 48, 72 and 96 hours in RPMI1640 media supplemented with 10% FBS, 1% penicillin and streptomycin. Effector cells obtained from suspension fraction of the MLTRs at 72 hours were harvested and incubated over night with [^{3}H]-thymidine (1 μCi/well). [^{3}H]-thymidine incorporation was detected using a □-counter (Packard, Meriden, CT, USA). Each experiment was performed in triplicate wells.

Flow cytometry analysis. The primary RMECs and RMTCs were labeled with FITC-conjugated hamster anti-mouse CD40 antibody (HM40-3, Becton Dickinson, San Jose, CA, USA). HM40-3 recognizes the N-terminal epitope of mouse CD40 and previously reported to be cross-reactive with Sprague-Dawley strain of rats.[35] CD40 expression levels were calculated with the mean fluorescence intensity (MFI) ratio of the CD40$^+$/CD40$^-$ cell populations. This antibody was also used for direct immunofluorescence staining of the primary cells.

Splenocytes derived from the suspension fraction of MLTRs were incubated with R-PE-conjugated mouse anti-rat CD80 (3H5, Becton Dickinson) or mouse anti-rat CD86 (24F, Becton Dickinson) antibodies. The cells were analyzed on an EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA, USA). Percentage of positive cells was calculated by comparison with appropriate isotype-matched antibody controls.

Quantitative RT-PCR analysis. Total RNA was isolated with TriReagent (Sigma). cDNA was synthesized from 2 μg of RNA, using oligo(dT) primers and RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to manufacturer's instructions. The primers designed for rat *β-actin, CD40, TNFα, IL-1β, CXCL1* and *IL-6* genes are listed in Table 1. *β-actin* was amplified as reference house keeping gene. PCR was carried out in a reaction mixture containing 1x LightCycler-DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany), 0.125 μM primer oligonucleotides and 3.5 mM $MgCl_2$ using the cycling conditions; 30" at 94°C, 30" at 60°C, 30" at 72°C (ABI Prism 7700 Sequence Detection System, Applied Biosystems, Foster City, CA, USA). Each reaction was performed in duplicates. Comparative Ct (ΔΔCt) method was used for the relative quantification of target gene expression.[36] Briefly, expression data of the gene of interest obtained from the experimental and control samples were first normalized with their *β-actin* gene expression and then the experimental samples are re-normalized with the control samples. Accordingly, the gene expression in RMTCs and in NMU cells was normalized against RMECs; pCD40L-t-RMTC and pCD40L-t-NMU data were normalized against pIRES2-t-RMTCs and pIRES2-t-NMU, respectively.

Statistical analysis. All values are expressed by arithmetic mean ± standard deviation (SD). Statistical difference between experimental groups was determined using student's paired or unpaired t-test where appropriate. Differences were regarded as statistically significant when $p \leq 0.05$.

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