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Chronic stress suppresses anti-tumor T_{CD8+} responses and tumor regression following cancer immunotherapy in a mouse model of melanoma

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ABSTRACT

Animal tumor models and human cancer studies have provided convergent evidence that chronic psychological stress plays a decisive role in modulating anti tumor T cell immunity. However, whether chronic stress also affects anti cancer vaccine strategies that rely on the induction of functional tumor specific T_{CD8+} cells has not been investigated yet. In this study we provide direct evidence that chronic stress suppresses the therapeutic efficacy of a biodegradable poly(D,L lactide co glycolide) microsphere (PLGA MS) based cancer vaccine in a murine melanoma model. Exposure of mice to social disruption stress (SDR), a well established model mimicking psychological chronic stress in humans, significantly impaired tumor protection in response to cancer vaccination under both prophylactic and therapeutic conditions. Vaccine failure in stressed mice correlated with significantly reduced generation of interferon γ (IFN γ) producing T_{CD8+} effectors and CTL mediated killing. Phenotypic analysis of dendritic cells (DCs) revealed that both migratory and lymphoid resident DCs failed to undergo full maturation upon antigen uptake. Notably, decreased DC maturation was associated with a significant impairment of peripheral DCs to migrate to draining LNs and to prime subsequent T_{CD8+} responses *in vivo*. In conclusion, chronic stress represents an important factor mediating immunosuppression in cancer vaccinated hosts by impairing DC functions and subsequent T_{CD8+} priming. Potentially, the mechanistic insights gained in this study open new avenues in utilizing the full potential of anti cancer vaccination strategies.

1. Introduction

Cancer vaccines are considered the most innovative immunotherapeutic strategies for treating or preventing the recurrence of malignancy, as they offer distinct advantages such as high specificity, reduced toxicity, and potential long term effects via immunological memory (Mellman et al., 2011). Several promising cancer vaccination strategies have been developed that are now studied in clinical trials for various cancer types (Guo et al., 2013). However anti tumor T_{CD8+} responses to vaccines currently tested in the clinic are often of low magnitude and many cancer patients do not establish sufficient T_{CD8+} effector cells necessary for tumor regression. To date immunosuppressive mechanisms limiting anti tumor responses are complex and not fully understood (Eng et al., 2014). In order to better realize the potential of anti cancer vaccine strategies, more efforts should be dedicated towards understanding the causes and mechanisms that impair vaccine induced anti tumor immunity.

Chronic psychological stress is associated with suppressed immune responsiveness and, as such, leads to increased susceptibility to malignancies, infections, and a poor response to vaccines (Segerstrom and Miller, 2004). Recent evidence reveals that chronic stress associated immunosuppression may also be of clinical significance for cancer disease and therapy (Eng et al., 2014; Antoni et al., 2006). For example, patients diagnosed with cancer are known to experience severely increased stress, and prior studies have found a strong correlation between stress and impaired T cell functions and NK cell cytotoxicity in patients with breast and ovarian cancer (Andersen et al., 1998; Lutgendorf et al., 2005; Thornton et al., 2007). Animal models using chronic stressors that promote fear or anxiety have provided further evidence for stress dependent suppression of tumor specific T_{CD8+} responses. For example, in a murine T cell lymphoma model immobilization stress suppressed tumor specific cytotoxic responses and secretion

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of interferon γ (IFN γ), accompanied by an accelerated tumor progression (Frick et al., 2009). Moreover, neuroendocrine stress mediators such as noradrenaline and adrenaline have been shown to inhibit *in vitro* generation of tumor specific cytotoxic T cells to transplanted MO 5 syngeneic tumors (Kalinichenko et al., 1999).

Apart from the cancer setting, we and others have shown that chronic stress in mice leads to significant dysregulations of T cell functions such as impaired cytotoxicity, decreased cytokine production, and a loss of the proliferative capacity upon T cell receptor (TCR) engagement (Sommershof et al., 2011). These effects ultimately result in diminished anti viral T_{CD8+} responses following infection (Sommershof et al., 2011; Elftman et al., 2010) and vaccination (Nezam et al., 2015; Tournier et al., 2001). Whether such stress dependent T cell impairment also affects anti cancer vaccine strategies relying on the induction of functional tumor specific T_{CD8+} cells has not been investigated yet.

Our present study was designed to investigate the effects and the mechanistic basis of chronic stress affecting a dendritic cell (DC) based cancer vaccine. As an efficient strategy to deliver vaccine components to DCs we utilized biodegradable microsphere (MS) prepared from poly(D,L lactic co glycolic acid, PLGA) that are biocompatible and biodegradable polymers that have FDA approval for human use (Waeckerle Men and Groettrup, 2005). The main advantages of using PLGA MS as a cancer vaccine is that they provide a non toxic, protective vehicle for co delivery of antigens and adjuvants that can be released over a sustained period of time. In addition, these particles are efficiently internalized by professional APCs, known as dendritic cells (DCs) which are of particular importance as DCs are potent initiators of antigen specific cytotoxic T_{CD8+} lymphocyte (CTL) responses. Subcutaneous immunization of mice using PLGA MS particles encapsulated with the tumor associated antigen ovalbumin (OVA) efficiently induces potent and sustained anti tumor T_{CD8+} responses (Schlosser et al., 2008) towards the OVA derived SIINFEKL peptide and long term protection against B16 expressing OVA melanoma (Mueller et al., 2011), providing a convenient model to evaluate the modulation of anti tumor immunity. In this tumor model we exposed mice to social disruption stress (SDR), a well established model of chronic stress (Avitsur et al., 2001; Stark et al., 2001) that mimics stress induced neuroendocrine responses in humans. Here, we report that chronic stress significantly impairs vaccine responses and tumor protection in a subcutaneous B16 OVA melanoma model through the suppression of SIINFEKL specific T_{CD8+} effectors. We further show that the compromised T_{CD8+} generation is due to an impaired ability of vaccine targeted DCs to infiltrate the draining lymph nodes (dLN) and to prime subsequent tumor specific T_{CD8+} responses. These findings highlight the critical role of chronic stress in modulating anti tumor T_{CD8+} responses and implicate DCs as main targets of stress *in vivo.*

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (H 2^b) were originally obtained from Charles River Laboratories. B6.PL (Thy1.1) congenic mice bearing the allelic variant of Thy1 (CD90) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used in adoptive transfer experiments (Experiment 3b). OT I mice were obtained from Dr. Ying Waeckerle Men (University of Zurich, Switzerland). Experimental mice were used at 7 8 weeks of age and were kept in a pathogen free facility on a 12/12 h light/dark cycle with *ad libitum* access to food and water. Both stressed and control mice were housed in groups of 5 mice per cage. All animal experiments were approved by the reviewing board of Regierungspräsidium Freiburg.

2.2. Stress procedure

The social disruption (SDR) procedure was described elsewhere and has been shown to induce chronic social stress in mice (Avitsur et al., 2001; Stark et al., 2001). The stress paradigm is based on the disruption of an established social hierarchy of group housed male mice (residents) which is experimentally induced by daily confrontations with an unfamiliar aggressive intruder mouse. A dominant intruder, previously screened for aggressive behavior, was introduced into the resident's cage for 2 h daily over a period of six consecutive days. To prevent habituation, a different aggressor was used for each stress cycle. The stress procedure always started at the beginning of the dark period when animals display increased activity and naturally rising glucocorticoid levels. Control mice were left undisturbed in their home cages throughout the entire experiment. SDR mice were carefully inspected after each stress cycle and mice that displayed bite wounds were taken out of the experiments.

2.3. Preparation of PLGA MS

MS were prepared from 14 kDa PLGA 50:50 carrying hydroxyl and carboxyl end groups (Resomer RG502H, Boehringer Ingelheim, Ingelheim, Germany). The antigens and TLR ligands were microencapsulated by spray drying as described elsewhere (Sabado and Bhardwaj, 2015). Briefly, 50 mg ovalbumin (Grade V, Sigma) and 5 mg CpG oligodeoxynucleotides with a phosphorothioate backbone (CpG ODN 1826, Microsynth, Switzerland) (MS Ova/CpG) or 0,5 mg polyinosinic polycytidylic acid (poly(I:C) (Calbiochem, VWR, Switzerland) (MS polyI:C) were dissolved in 0.5 ml 0.1 M NaHCO3 (aqueous phase) and mixed with 1 g of PLGA dissolved in 20 ml of dichloromethane (organic phase), emulsified by ultrasonication and immediately spray dried (Büchi, Mini Spray Dryer 191) at a flow rate of 2 ml/min and inlet/outlet temperatures of 40/37 °C. Immediately before use PLGA MS were dispersed in PBS by ultrasonication for 30 s.

2.4. Tumor cell line and tumor inoculation

The B16 melanoma is a tumor of spontaneous origin in C57BL/6 mice. The OVA transfectant of the C57Bl/6 derived B16 melanoma, MO 5, was originally generated by transfection of B16 melanoma with plasmid pAc neo OVA and was kindly provided by Dr. Antje Heit (LMU Munich). MO 5 cells were maintained at 37 °C and 5% CO2 in DMEM media supplemented with GlutMAX, 10% FCS, 100 U/ml of penicillin/streptomycin and 0.4 mg/ml G148 to maintain OVA expression. Cells were split two days prior to subcutaneous injection to ensure consistent growth. For the inoculation of mice, a number of 5×10^5 MO 5 cells, which we titrated to yield optimal tumor growth, were injected subcutaneously into the right flank.

2.5. Tumor immunization

Mice were immunized either with a mixture of 5 mg PLGA MS, containing ovalbumin (OVA) (250 μg) and CpG ODN (25 μg) (MS Ova/CpG) and 5 mg PLGA MS containing polyI:C (2,5 μg) (MS polyI:C) or the corresponding amounts of OVA, CpG ODN, and polyI:C in PBS:IFA (1:1) (in IFA). Control mice were either treated with empty MS (empty) or left untreated (naïve). All injections were performed by subcutaneous injection in the base of the tail in a total volume of 200 μl.

2.6. Immunization with $CD11c^+$ DCs

The capacity of $CD11c^+$ DCs to activate SIINFEKL specific T_{CD8+} cells *in vivo* was assessed. On day three after PLGA MS OVA immu

nization, spleens were collected and CD11c positive DCs were enriched by positive selection using a $CD11c^+$ Cell Isolation Kit (Miltenyi Biotec, Germany). Subsequently, OT I recipient mice were immunized by intravenously transfer of 4×10^6 sorted $CD11c^+$ cells. For some experiments sorted $CD11c^+$ cells were externally pulsed with 1×10^6 M SIINFEKL peptide for 1 h at 37 °C before adoptive transfer. Five days after $CD11c^+$ transfer, spleens from OT I mice were removed and splenocytes were ana lyzed for SIINFEKL specific T_{CD8+} generation by performing an intracellular cytokine staining (ICS) for IFN γ.

2.7. Adoptive T cell transfer

To better understand the mechanisms of impaired CTL responses in SDR mice, Thy1.1 transgenic B6.PL mice were used as SDR mice to monitor proliferation of i.v. transferred SIINFEKL specific OT I donor T cells *in vivo*. In this model transferred Thy1.2+ T_{CD8+} cells are identified by flow cytometric analysis using mAb to discern allotypic differences between TCR transgenic mice and donor mice in the cell surface molecule Thy1. Briefly, Thy1.1 mice were subjected to six days of SDR and were subsequently vac cinated with PLGA MS OVA/CpG and MS polyI:C as detailed previ ously (Mueller et al., 2011). On day three post immunization, splenocytes from naïve OT I donor mice were magnetically sorted by positive selection using a $CD8a^+$ T Cell Isolation Kit II (Miltenyi Biotec, Germany) and cells were labeled with 10 μM carboxyfluo rescein diacetate succinimidyl ester (CFSE). 5×10^6 purified T_{CD8+} cells were intravenously (i.v.) transferred into SDR or control Thy1.1 recipient mice which had been immunized with PLGA MS OVA 3 days before. 44 h after the transfer, spleen cells were collected and the proliferation of transferred OT I Thy1.2 cells was measured via CFSE dilution by flow cytometry.

2.8. In vivo cytotoxicity assay

To determine the cytolytic activity of OVA specific $CD8^+$ T cells *in vivo*, killing of epitope specific CFSE labeled transferred target cells was investigated. Briefly, syngeneic naive C57BL/6 spleno cytes were used as target cells and pulsed for 1 h at 37 °C with SIINFEKL peptide at 2 μM in RPMI medium or were incubated without peptide. Cells were washed twice with PBS and were labeled with either 5 μM CFSE (SIINFEKL pulsed; $CFSE^{high}$ cells) or 0.5 μM CFSE (unpulsed, $CFSE^{lo}$ cells) by incubation for 10 min at 37 °C in PBS 0.1% FCS. A mixture of $5x10^6$ $CFSE^{high}$ and $5x10^6$ $CFSE^{lo}$ cells was injected intravenously into day 5 immunized con trol and SDR mice. Splenocytes of recipient mice were analyzed 18 h later for quantification of $CFSE^-$ labeled cells. The percentage of specific killing was calculated by the difference between $CFSE^{high}$ cells and $CFSE^{lo}$ in immunized control and SDR vs non immunized mice.

2.9. In vitro Antigen Presentation assay

In order to gain mechanistic insights into the cause of the com promised ability of chronically stressed mice to prime SIINFEKL specific T_{CD8+} cells, we examined the overall capacity of isolated $CD11c^+$ cells to prime an autologous T_{CD8+} line established from OT I mice infected with a recombinant vaccinia virus (rVV) expressing SIINFEKL. Spleen cells were collected from control and SDR mice on the last day of the stress procedure. Subsequently, $CD11c^+$ cells were enriched by positive selection using a $CD11c^+$ Cell Isolation Kit (Miltenyi Biotec, Germany). $CD11c^+$ DCs were incubated with PLGA MS OVA/CpG at 37 °C overnight, to allow uptake and processing of encapsulated OVA peptide. Cells were collected the next day and used as stimulator cells in an antigen presentation assay. A SIINFEKL/H $2K^b$ specific CTL line was gener ated from OT I mice which had been infected with rVV SIINFEKL as previously described (Basler et al., 2006) and used in the antigen presentation assay at an effector to stimulator (E:S) ratio of 1:2 and 1:1. In a second approach, $CD11c^+$ DCs were pulsed with 10^{-7} M of SIINFEKL peptide for 1 h at 37 °C, washed three times with PBS and used as stimulator cells. Direct antigen presentation was measured using IFN γ ICS.

2.10. Intracellular cytokine staining (ICS) for IFN γ

ICS was performed to quantify vaccine specific cytokine pro duction by T_{CD8+} cells. Briefly, splenocytes (2×10^6 cells) were incubated in 96 well round bottom plates with 10^{-7} M of SIINFEKL peptide in 100 μl IMDM/10% FCS in the presence of bre feldin A (10 μg/ml) for 5 h at 37 °C. Cells were stained with Cy5 conjugated anti mouse CD8α (clone 53 6.7, BD PharMingen, San Diego, CA) for 20 min at 4 °C. Following fixation with 4% paraformaldehyde at 4 °C for 5 min, cells were incubated overnight with FITC conjugated anti mouse IFN γ (clone XMG1.2 BD PharMingen) in PBS containing 2% FCS and 0.1% (w/v) saponin (Sigma, Germany). The following day, samples were washed twice and acquired on a FACScan flow cytometer and analyzed using FlowJo software.

2.11. Pharmacologic depletion of Circulating Macrophages

Clodronate liposomes for macrophage depletion were pur chased from Nico van Rooijen (Vrije University, Amsterdam, Netherlands). Macrophage depletion was performed by intra venous and subcutaneous tail injection of 200 ml clodronate encapsulated in liposomes. This application has previously been shown to result in the depletion of macrophages in the blood, spleen, liver, bone marrow as well as the draining lymphatics (Schliehe et al., 2011). The effective depletion of macrophages was confirmed by staining of splenocytes for F4/80 and flow cytometry.

2.12. Plasma corticosterone (CORT) measurement

Hormone levels in mouse plasma were measured on day 4 of the SDR procedure using enzyme linked immunosorbent assay (ELISA) method. A corticosterone ELISA kit (ADI 900 097, Enzo Life Science, NY, USA) was used according to the manufacturer's instructions. Mouse blood from Vena facialis was rapidly collected into chilled heparinized tubes and kept on ice. Plasma was drawn off after centrifugation at 2000g for 5 min at 4 °C. Samples were stored at 80 °C until processing. Briefly, plasma levels of bound, unbound, and total CORT were quantified as following: 10 μl dupli cate samples of plasma from each mouse were diluted with 10 μl 1:100 Steroid Replacement Reagent (final concentration 1:40). This enabled measurement of bound CORT. Directly after adding the stop solution to each well, the optical density (OD) was read at 450 nm with correction at 585 nm using a microplate reader (Tecan). CORT concentrations were expressed as pg/mL of plasma after being fit to a 4 parameter logistic regression standard curve.

2.13. Statistical analysis

Data are expressed as mean ± S.E.M. Means of two independent groups were analyzed using Student's *t* test for independent pairs. A two factor ANOVA was used to test for the effects of stress and drug treatment as well as the interaction of both factors. The level of significance was set at $p < 0.05$. All statistics were calculated using GraphPad InStat 3 for Windows (GraphPad Software, La Jolla, CA).

3. Results

3.1. Chronic stress inhibits PLGA MS induced tumor protection and regression of established tumors

In order to elucidate the effects of chronic stress on the efficacy of PLGA MS based immunotherapy, we assessed the effects chronic social disruption stress on tumor growth and overall sur vival in a protective (Fig. 1A) and therapeutic (Fig. 1B) tumor set ting. As shown in Fig. 1A, stressed mice exhibited a strikingly reduced tumor protection in response to PLGA MS OVA vaccina tion relative to non stressed control mice (Control, $n = 4$; SDR, $n = 6$; $p = 0.0005$). While immunized control mice were completely protected against the tumor challenge, SDR mice developed stea dily progressing palpable tumors and had to be euthanized between day 16 and day 24, respectively. The same outcome was observed in the therapeutic setting (Fig. 1B), in which stressed and control groups were challenged with ovalbumin expressing B16 melanoma cells (MO 5) at the onset of the SDR procedure and immunotherapy with PLGA MS OVA was started as soon as palpable tumors had occurred. PLGA MS OVA mediated rejection of MO 5 tumors was apparent in control mice between days 7 and 10 and was complete on day 14. In contrast, PLGA MS OVA immunized stressed mice could not be protected from B16 tumors and 80% died within 30 days (Fig. 1B). (Control, $n = 5$; SDR, $n = 5$; $p = 0.0039$).

3.2. Vaccine specific T_{CD8+} responses are significantly reduced in chronically stressed mice

Because T_{CD8+} cells are known to play a major role in vaccine mediated tumor rejection, we determined whether chronic stress would influence the generation of tumor specific T_{CD8+} cells. Mice were exposed to social disruption stress and subsequently vacci nated with PLGA MS OVA. H $2K^b$/SIINFEKL tetramer staining was performed to quantify OVA specific T_{CD8+} cells in the spleen at day six post vaccination. As shown in Fig. 2A, chronic stress expo sure before PLGA MS vaccination significantly reduced the fre quency of tetramer specific T_{CD8+} cells (control: $1.6 \pm 0.1\%$, $n = 20$ vs. SDR: $0.98 \pm 0.1\%$, $n = 22$; $t(40) = 3.8$; $p = 0.0004$). We further quantified IFN γ secreting SIINFEKL specific T_{CD8+} cells by per forming ICS and enzyme linked immunospot (ELISpot) assays. As shown in Fig. 2, chronically stressed mice exhibit a significant reduction in the percentage (Fig. 2B, control: $0.53 \pm 0.1\%$, $n = 15$ vs. SDR: $0.32 \pm 0.1\%$, $n = 14$; $t(27) = 6.2$; $p = 0.0001$) and absolute number (Fig. 2D, control: 346 ± 25, $n = 5$ vs. SDR: 147 ± 36, $n = 5$; $t(8) = 4.6$; $p = 0.002$) of IFN γ producing antigen specific T_{CD8+} cells compared to non stressed control mice. Notably, a vaccine formu lation containing a mixture of incomplete Freund's Adjuvant (IFA), OVA and CpG also induced significantly less vaccine specific T_{CD8+} cells in stressed mice (Fig. 2C, control: $2.0 \pm 0.1\%$, $n = 4$ vs. SDR: $1.1 \pm 0.2\%$, $n = 5$; $t(7) = 3.5$; $p = 0.01$), suggesting that stress induced impairment of anti tumor T_{CD8+} cell generation is also rel evant for other tumor vaccination approaches.

We next examined the effector phenotype of the generated T_{CD8+} cells specific for the OVA peptide SIINFEKL with a particular emphasis on markers that are associated with anti tumor effector function, including CD44, the lymphocyte homing receptor CD62L and the killer cell lectin like receptor G1 (KLRG1). We found that the frequencies of $CD62L^{low}/CD44^{high}$ cells among the tetramer positive T_{CD8+} cells were comparable between the SDR and control group (data not shown). However, the formation of terminally dif ferentiated effector T_{CD8+} cells (defined by a $KLRG1^+$ phenotype)

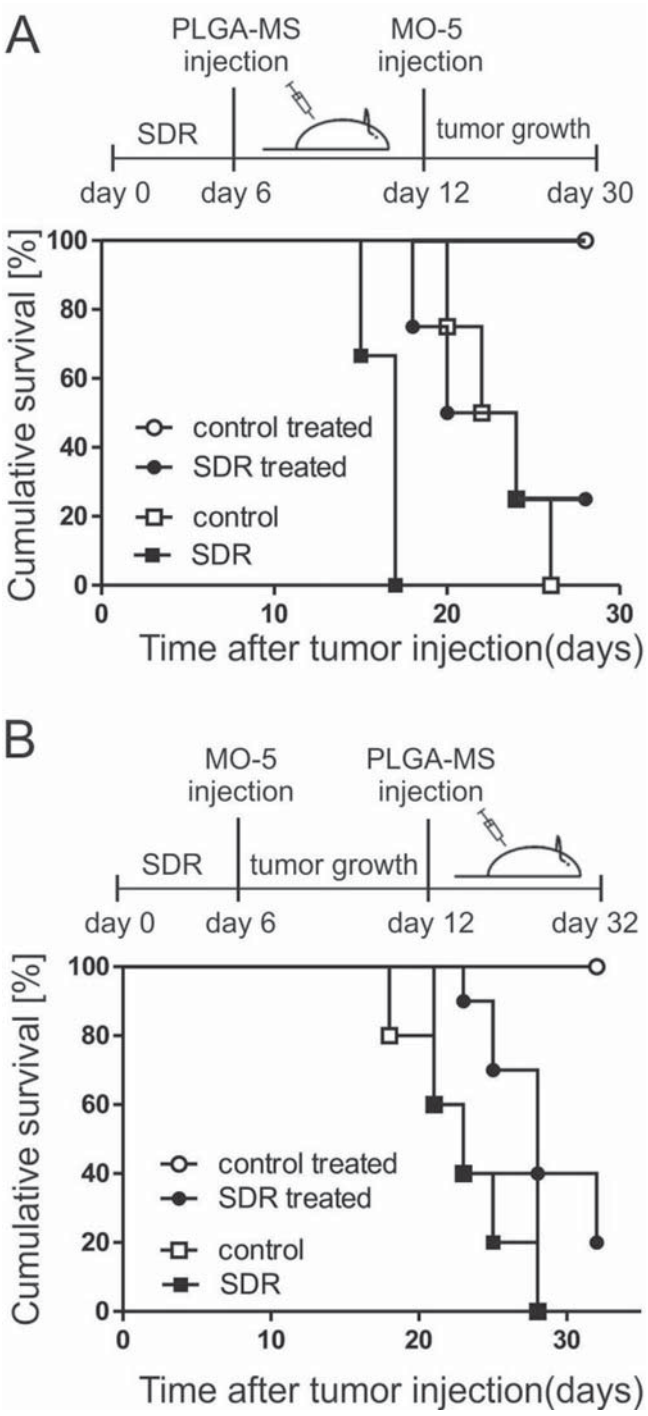

Fig. 1. Chronic stress inhibits PLGA-MS-induced tumor protection and regression of established tumors. Survival of tumor-bearing SDR and control mice in a (A) protective and (B) therapeutic tumor setting. C57BL/6 mice were subjected to social disruption stress or were left undisturbed in their home cages. (A) On the last day of the stress procedure, SDR and control mice were immunized with PLGA-MS-OVA/CpG and MS polyI:C. Six days after vaccination mice were challenged with B16 melanoma expressing ovalbumin (OVA). Mice were monitored for onset of tumor development and tumor sizes were measured daily until they reached 15 mm in mean size of 2 orthogonal measurements. Control, $n = 4$; SDR, $n = 6$. (B) On the last day of the stress procedure, SDR and control mice were challenged with MO-5 cells. Treatment of mice by PLGA-MS-OVA/CpG and PLGA-MS-polyI:C immunization was started individually as soon as palpable tumors occurred. Tumor sizes were measured daily until they reached 15 mm in mean size of 2 orthogonal measurements. Control, $n = 5$; SDR, $n = 5$. Data are representative results from two independent experiments.

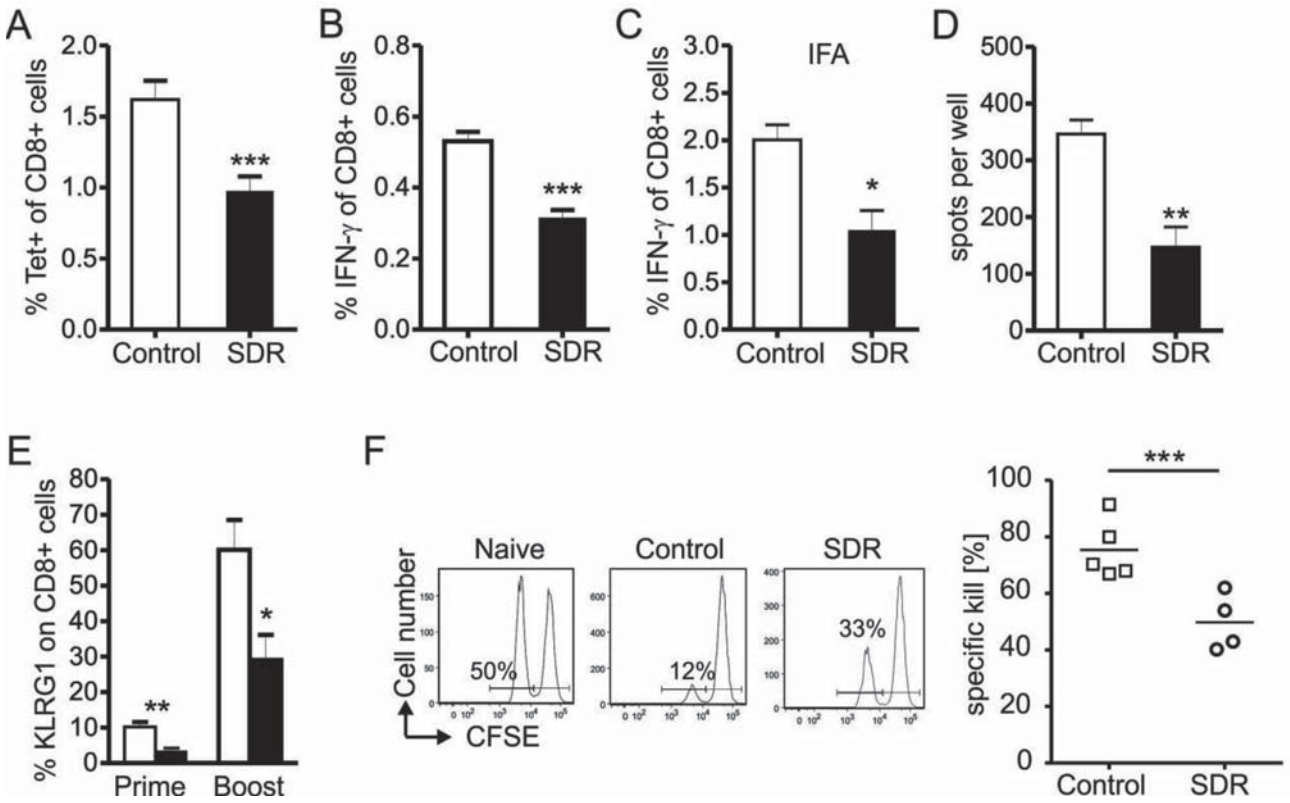

Fig. 2. Chronic stress impairs antigen-specific T_{CD8+} effector cell generation after PLGA-MS immunization. Mice were subjected to SDR and were subsequently immunized with PLGA-MS OVA/CpG and MS polyI:C. Vaccine-*specific effector* T_{CD8+} *generation* was analyzed in SDR (filled columns) and control (open columns) mice on day 6 post vaccination. (A) SIINFEKL-specific T_{CD8+} cell generation was determined by H-2K^b/SIINFEKL-tetramer staining. Bar graphs indicate the mean percentage of H-2K^b/SIINFEKL-tetramer T_{CD8+} cells (±SEM) within the total T_{CD8+} population. Control, $n = 20$; SDR, $n = 22$. (B, C) IFN-γ ICS was performed to quantify the frequency of IFN-γ secreting T_{CD8+} cells after *in vitro* restimulation in the presence of SIINFEKL peptide. Bar graphs represent the mean percentage of IFN-γ secreting T_{CD8+} cells (±SEM) within the total T_{CD8+} cell population after immunization with (B) PLGA-MS OVA/CpG and MS polyI:C. Control, $n = 15$; SDR, $n = 14$, or (C) OVA, CpG and poly(I:C) in IFA, Control, $n = 4$; SDR, $n = 5$. (D) Numbers of IFN-γ secreting T_{CD8+} cells were quantified by cytokine-release ELISpot assay. Bar graphs represent the mean number of IFN-γ secreting T_{CD8+} cells (±SEM), Control, $n = 5$; SDR, $n = 5$. (E) On day 6 after the first immunization (Prime) and after booster immunization (Boost) cells were stained with H-2K^b/SIINFEKL tetramers and KLRG1 to assess the numbers of fully differentiated effector T_{CD8+} cells. Bar graphs indicate the mean percentage of KLRG1$^+$ cells (±SEM) within the total H-2K^b/SIINFEKL T_{CD8+} population, Control, $n = 17$; SDR, $n = 15$. (F) *In vivo* CTL assay. Cytolytic activity of OVA-specific CD8$^+$ T cells was measured in day 5 immunized Control and SDR or unimmunized mice (Naïve) by flow cytometry 18 h after mice were injected with CFSE^{lo} SIINFEKL-pulsed and CFSE^{high} unpulsed splenocytes at a ratio of 1:1, Control, $n = 9$; SDR, $n = 8$. A representative histogram plot from each group is shown. *In vivo* significance is indicated with $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

was significantly lower in the SDR group (Fig. 2E; control: 10.21 ± 1.3%, $n = 17$ vs. SDR: 3.57 ± 0.7%, $n = 15$; $t(30) = 4.2$; $p = 0.0002$). Moreover, booster vaccination seven days after the first vaccination resulted in a pronounced increase in the proportion of terminally differentiated KLRG1 expressing cells among tetramer positive T_{CD8+} cells. Still, the ability of Ag specific T_{CD8+} cells to acquire a fully differentiated KLRG 1^{hi} effector phenotype was significantly altered in SDR mice (Fig. 2E; control: 60.1 ± 8.4%, $n = 5$ vs. SDR: 29.1 ± 7.1%, $n = 5$; $t(8) = 2.8$; $p = 0.02$). To directly compare the cytolytic activity of CTLs generated in SDR mice with those of unstressed control mice, we measured *in vivo* killing of transferred SIINFEKL pulsed target cells in day 5 PLGA MS immunized mice. As shown in Fig. 2F, SDR mice demonstrated a significantly reduced killing of target cells compared to control mice (control: 74.4 ± 4.4%, $n = 9$ vs. SDR: 39.7 ± 6.2%, $n = 8$; $t(15) = 4.7$; $p = 0.0003$). Collectively our data show that chronic stress affects the formation of antigen specific T_{CD8+} effectors, preventing CTL killing of the tumor cells. This observation may contribute significantly to the impaired vaccine responses and tumor protection in chronically stressed mice.

3.3. Chronic stress affects the APC compartment rather than intrinsic T cell function

Poor T cell responses can be attributed mainly to either intrinsic defects in the T cell population or impaired T cell priming by antigen presenting cells (APCs). To investigate the possibility of an intrinsic T cell defect, mice were exposed to social disruption stress, subsequently vaccinated with SIINFEKL pulsed BMDCs and six days later IFN γ secretion of SIINFEKL specific T_{CD8+} cells were quantified by ICS. As shown in Fig. 3A, the number of IFN γ producing T_{CD8+} cells generated following intravenous vaccination with peptide BMDCs, was not reduced in stressed mice (control: 1.3 ± 0.2%, $n = 5$ vs. SDR: 1.3 ± 0.3%, $n = 5$; $t(8) = 0.11$; $p = 0.911$). This observation suggests that the lowered levels of vaccine specific T cell responses observed in SDR mice were not due to any intrinsic T_{CD8+} defects.

We next examined the impact of stress on the ability of APCs to prime and expand antigen specific T_{CD8+} cells *in vivo*. SDR and control recipient mice were injected with CFSE labeled transgenic H 2Kb/SIINFEKL specific OT I T_{CD8+} cells at day three post PLGA MS OVA immunization and proliferation was determined 44 h later by CFSE dilution. As shown in Fig. 3B, proliferation of transferred OT I T_{CD8+} cells was significantly lower in SDR mice (Control: 64.8 ± 4.8%, $n = 4$ vs. SDR: 39.6 ± 4.4%, $n = 3$; $t(5) = 3.7$, $p = 0.01$). Similarly, when we adoptively transferred bulk LN cells derived from day three PLGA MS immunized mice and determined their overall priming capacity in OT I recipient mice, APCs from SDR mice were much less efficient in activating SIINFEKL specific T_{CD8+} cells in OT I recipient mice compared to control mice (Fig. 3C; control: 13.1 ± 1.9%, $n = 5$ vs. SDR: 5.3 ± 1.2% $n = 4$; $t(7) = 3.1$, $p = 0.016$). These results indicate that the failure of stressed

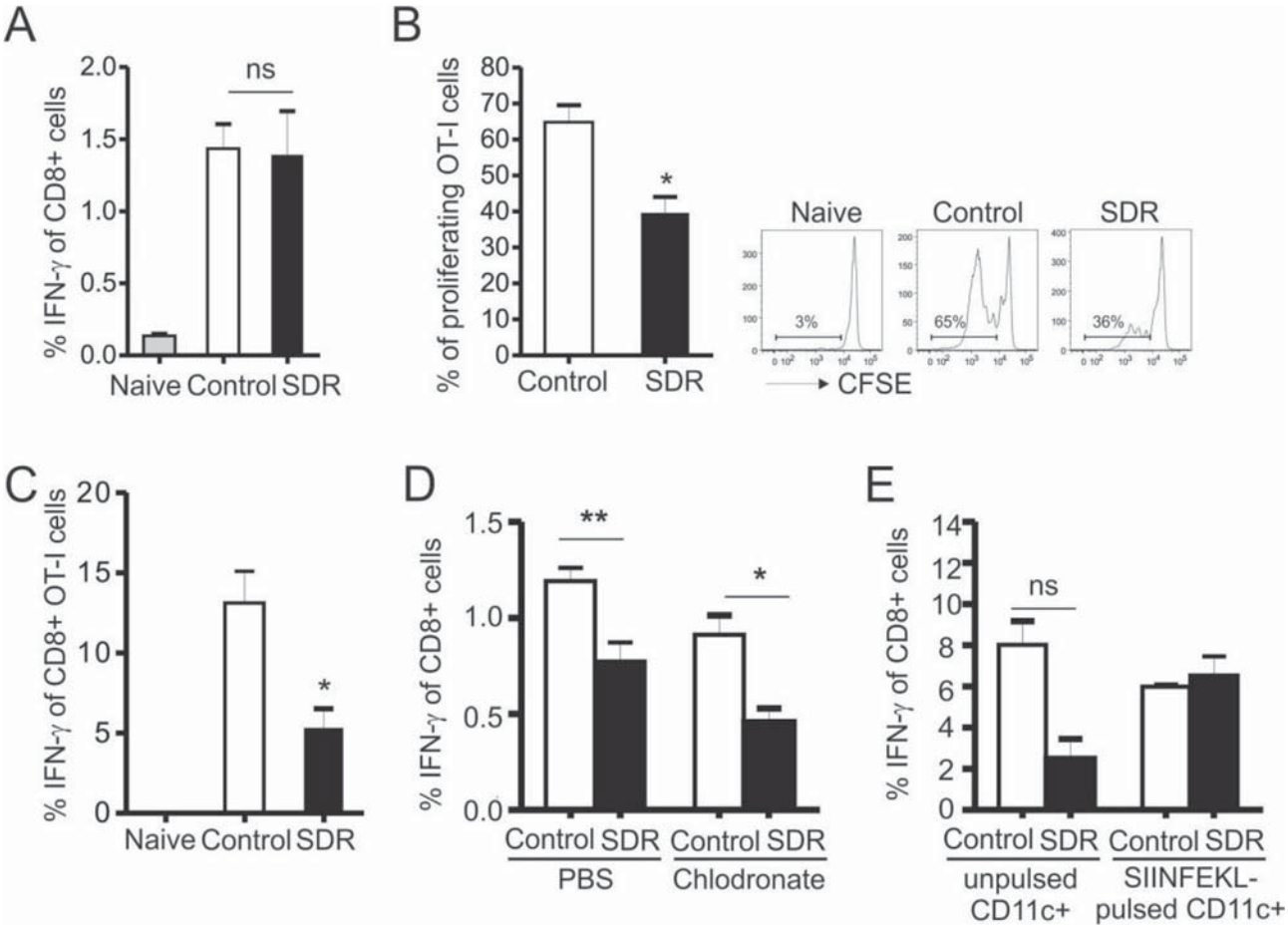

Fig. 3. Delineating the role of intrinsic T cell defects versus antigen presenting cell (APC) dysfunction *in vivo*. (A) T_{CD8+} *generation in control and SDR mice* immunized with SIINFEKL-pulsed BMDCs. SDR and control mice were immunized by i.v. injection of $2x10^6$ SIINFEKL-pulsed BMDCs and six days later IFN-γ -producing T_{CD8+} cells were quantified by ICS. Bar graphs represent the mean percentage of IFN-γ -producing T_{CD8+} cells (±SEM) within the total T_{CD8+} population. Control, $n = 5$; SDR, $n = 5$. (B) Proliferation of transferred naïve OT-I cells in immunized control and SDR mice. On day three after immunization with PLGA-MS-OVA/CpG and PLGA-MS-polyI:C control and SDR mice (Thy1.1) were injected with naïve CFSE-labeled OT-I cells (Thy1.2) and T cell proliferation was determined 44 h later by CFSE dilution. Control, $n = 4$; SDR, $n = 3$. Representative profiles gated on recovered $Thy1.2^+$ $OT\text{-}I^+$ cells show sustained OT-I proliferation in unstressed control mice compared with that observed in SDR mice. Bar graphs represent the mean percentage of proliferated OT-I cells (±SEM) in control, SDR or unimmunized (Naive) recipient mice. (C) The overall capability of bulk LN cells to prime antigen-specific T_{CD8+} cells was determined. SDR and control mice were vaccinated with PLGA-MS as used in (B) into both hind footpads. Three days later, popliteal LNs were harvested and the obtained single cell suspensions were i.v. injected into naïve recipient OT-I mice. OT-I mice were analyzed for IFN-γ -producing T_{CD8+} cells by ICS. Bar graphs represent the mean percentage of IFN-γ -producing T_{CD8+} cells (±SEM) within the total T_{CD8+} population. Control, $n = 5$; SDR, $n = 4$. (D) Effects of *in vivo* macrophage depletion on the T_{CD8+} priming capacity in SDR mice. On the last day of SDR procedure macrophages were depleted by injections of clodronate liposomes. Mice were immunized with PLGA-MS as in (B) and IFN-γ -producing T_{CD8+} cells were determined by ICS. Bar graphs represent the mean percentage of IFN-γ producing specific T_{CD8+} cells (±SEM) within the total T_{CD8+} population. Control, $n = 5$; SDR, $n = 4$. (E) Priming ability of $CD11c^+$ cells. Purified $CD11c^+$ cells from stressed and control mice on day 3 after PLGA-MS based immunization were pooled and either directly i.v. transferred into naive recipient OT-I mice or were pulsed with SIINFEKL-peptide before adoptive transfer. The overall priming capacity of transferred $CD11c^+$ DCs was analyzed five days later by IFN-γ ICS. Bar graphs represent the mean percentage of IFN-γ producing SIINFEKL-specific T_{CD8+} cells (±SEM) within the total T_{CD8+} population. Control, $n = 5$; SDR, $n = 5$. Shown are representative results from two independent experiments. Significance is indicated with $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

mice to mount a proper antitumor T_{CD8+} response is localized within the APC compartment.

As we have previously established that $CD11c^+$ DCs and macrophages (MΦs) are the two major APCs involved in the uptake and cross presentation of PLGA MS encapsulated antigen *in vivo* (Schliehe et al., 2011), we aimed at discriminating the contribution of each APC subpopulation on impaired T cell priming in SDR mice. We examined whether selective depletion of macrophages at the time of vaccination could affect stress induced changes of T_{CD8+} cell priming. Mice were treated 18 h before PLGA MS immunization with clodronate liposomes to achieve transient depletion of macrophages in the draining lymph nodes, blood, and spleen (Schliehe et al., 2011). SIINFEKL specific T_{CD8+} generation was analyzed six days post vaccination by ICS. Depletion of macrophages did not reverse the stress induced decline of responding T_{CD8+} cells (Fig. 3D, control: 0.91 ± 0.1%, $n = 4$ vs SDR: 0.45 ± 0.8%%, $n = 4$; $t(7) = 3.5$, $p = 0.01$), indicating that macrophages are not the main targets of the stress response. To examine if stress impaired the ability of DCs to process and present cognate peptides to antigen specific T_{CD8+} cells, we assessed the capacity of isolated $CD11c^+$ DCs from day three PLGA MS immunized mice to activate a SIINFEKL specific T cell response in untreated OT I recipient mice. As shown in Fig. 3E, purified splenic $CD11c^+$ DC cells derived from SDR mice had a substantially lower ability to stimulate a T_{CD8+} cell response in OT I recipient mice compared to equal numbers of $CD11c^+$ DC originating from unstressed mice (Fig. 3E, control: 8.0 ± 1.2%, $n = 2$ vs. SDR: 2.5 ± 0.9%, $n = 2$; $t(2) = 3.7$, $p = 0.07$). We validated these results and modified the experimental set up by externally pulsing $CD11c^+$ DCs from stressed and control mice with the K^b binding OVA epitope SIINFEKL before adoptive transfer and investigated whether the attenuated CTL induction is still present in OT I recipient mice. As shown in Fig. 3E, no differences in

T_{CD8+} responses were detected. Collectively, our data presented so far support the concept that a compromised priming capacity of $CD11c^+$ cells impedes optimal anti tumor T_{CD8+} induction in chron ically stressed mice.

3.4. Chronic stress significantly alters the potential of DCs to mature and to present PLGA MS encapsulated antigen for subsequent T_{CD8+} priming

In order to gain mechanistic insights into the cause of the com promised ability of chronically stressed mice to prime SIINFEKL specific T_{CD8+} cells, we examined critical steps involved in the anti gen presentation process *in vitro*: antigen uptake, peptide presen tation and DC maturation. Initially, we investigated the overall capacity of isolated $CD11c^+$ cells to prime an autologous T_{CD8+} line established from OT I mice infected with a recombinant vaccinia virus (rVV) expressing SIINFEKL. Isolated $CD11c^+$ DCs from naïve stressed and control mice were incubated with PLGA MS overnight and co cultured with SIINFEKL specific CTLs. IFN γ producing T_{CD8+} cells were then quantified by ICS. As shown in Fig. 4A, $CD11c^+$ cells from SDR mice have an inferior ability to activate SIINFEKL specific T_{CD8+} cells compared to $CD11c^+$ cells from control mice (Fig. 4A, control: 4.95 ± 0.3%, $n = 5$ vs SDR: 3.39 ± 0.1%, $n = 5$; $t(8) = 4.9$, $p = 0.001$). External peptide pulsing of $CD11c^+$ DCs with the H $2K^b$ binding OVA epitope SIINFEKL rescued the attenuated CTL induction. In order to rule out the possibility of insufficient antigen uptake, we next analyzed the ability of APCs to actively internalize PLGA MS encapsulated antigens. Purified splenic cells from stressed and control mice were monitored for their capacity to take up fluorescent quantum dot (QD) labeled PLGA MS *in vitro* by flow cytometry. As shown in Fig. 4B, internalization of QD bearing PLGA MS varied between the $CD11c^+$, $CD11b^+$, $F4/80^+$, and the MHC class II^+ population but did not differ significantly between SDR and control mice.

Because effective T cell activation is dependent on the matura tion stage of DCs, we addressed the possibility that chronic stress exposure impacts DC maturation. Purified $CD11c^+$ cells from stressed and control mice were co cultured in the presence of PLGA MS OVA/CpG and typical signs of DC maturation were mon itored. As shown in Fig. 4C, the uptake of PLGA MS induced a 2 3 fold increase in mean expression of CD86, CD40, MHCII, and CCR7 on $CD11c^+$ cells derived from control mice within 24 h. However, the extent of DC maturation, as reflected by upregulation of cos timulatory (CD86, CD40) and MHC II molecules as well as the che mokine receptor CCR7 was significantly reduced on $CD11c^+$ cells from stressed mice.

Various studies have suggested a role for stress induced corti costerone (CORT) secretion in modulation of DCs function and mat uration (Elftman et al., 2007). To verify individual stress responses and their effect on DC maturation, we determined plasma CORT levels and atrophy of the thymus, both being the consequence of frequent adrenocortical activation and a hallmark of the SDR model. As shown in Fig. 4D, repeated exposure to the social stres sor resulted in a significant rise in CORT levels, accompanied by a marked reduction of thymus mass in SDR mice that ultimately impeded phenotypic maturation of $CD11c^+$ DCs after LPS stimula tion. Collectively, these data provide evidence that CORT induced intrinsic DC dysfunction is a primary mechanism by which social stress impairs the efficacy of cancer vaccines.

3.5. Chronic stress impairs migration of Ag bearing DCs to the draining LNs

In order to investigate whether the poor maturation influences the capacity of antigen loaded DCs to migrate to the dLN we injected fluorescent QD labeled PLGA MS into the footpad of SDR and control mice. At different time points after injection dLN were assessed for the total number of QD bearing by FACS analysis (Fig. 5A). A significantly lower rate of QD positive $CD11c^+$ cells reached LNs in SDR mice after 24 h (control: 170 ± 23, $n = 4$ vs SDR: 84 ± 20, $n = 5$; $t(7) = 2.9$, $p = 0.022$), 48 h (control: 722 ± 80, $n = 9$ vs SDR: 445 ± 83, $n = 6$; $t(13) = 2.3$, $p = 0.038$) and 72 h (con trol: 2195 ± 190, $n = 13$ vs SDR: 1042 ± 105, $n = 12$; $t(23) = 5.2$, $p = 0.0001$), suggesting that the efficiency of DCs migrating to lymph nodes was impaired after SDR exposure.

In order to rule out that the reduced number of QD bearing $CD11c^+$ cells reaching the draining LN is due to insufficient antigen uptake at the injection site, we next monitored the ability of APCs to actively internalize QD bearing PLGA MS antigens after subcu taneous footpad injection. As shown in Fig. 5B, the internalization of QD bearing PLGA MS *in vivo* did not differ between SDR and control mice. Collectively, these data provide evidence that the compromised T_{CD8+} generation is due to an impaired ability of vaccine targeted DCs to infiltrate the dLN and to prime subsequent tumor specific T_{CD8+} responses.

4. Discussion

Despite continued progress in the treatment of many tumors, cancer is one of the most stressful, life threatening illnesses that a patient might have to cope with. Accumulating evidence suggests that such chronic stress suppresses anti tumor T_{CD8+} immunity in cancer patients and animal models. However, whether stress adversely affects anti tumor T_{CD8+} efficacy of cancer vaccines has not been investigated yet. Here we show that chronic stress signif icantly impairs vaccine specific T_{CD8+} generation and tumor pro tection in a subcutaneous B16 ovalbumin (MO 5) melanoma model by impairing DC maturation and subsequent T_{CD8+} priming.

We have previously demonstrated that PLGA MS vaccination induced prophylactic as well as therapeutic protection against aggressive B16 (OVA) melanomas (Mueller et al., 2011). Here we found that mice undergoing six days of social disruption stress dis played a strikingly reduced tumor protection in response to immu nization under both prophylactic and therapeutic conditions. Chronically stressed mice exhibited significantly decreased num bers of vaccine specific, IFN γ producing T_{CD8+} effectors (Fig. 2A D). Moreover a significant fraction of the generated CTLs failed to acquire a fully matured effector phenotype, indicated by a low expression of KLRG 1 (Fig. 2E). The importance of T_{CD8+} effec tors for immune mediated tumor eradication in cancer patients is well established, and the generation of anti tumor T_{CD8+} cells is also a hallmark of tumor regression following PLGA MS vaccina tion (Waeckerle Men and Groettrup, 2005). Thus we reasoned that differences in tumor control are caused by changes in numbers and quality of vaccine induced T_{CD8+} cells. This assumption is further substantiated by the observation that chronically stressed mice showed a significant reduction of CTL mediated target cell killing.

DCs play a central role in the induction of anti tumor T_{CD8+} responses and they have been proven frequently as crucial factor for effective anti tumor control and the success of cancer immunotherapy (Palucka and Banchereau, 2012). Our findings reveal that the blunted T_{CD8+} response in the stressed animals was at least in part due to a blockade of DC maturation. Unlike DCs from control mice that responded to PLGA MS uptake by sig nificant up regulation of maturation associated factors such as CD40, CD86, and MHC class II receptors, DCs from chronically stressed mice showed considerable impairments in upregulating these maturation markers (Fig. 4C). Modulation of DC maturation and function by acute and chronic stress hormones has been described previously (Truckenmiller et al., 2006), suggesting that this is a general mechanism how chronic stress impedes T_{CD8+} cell

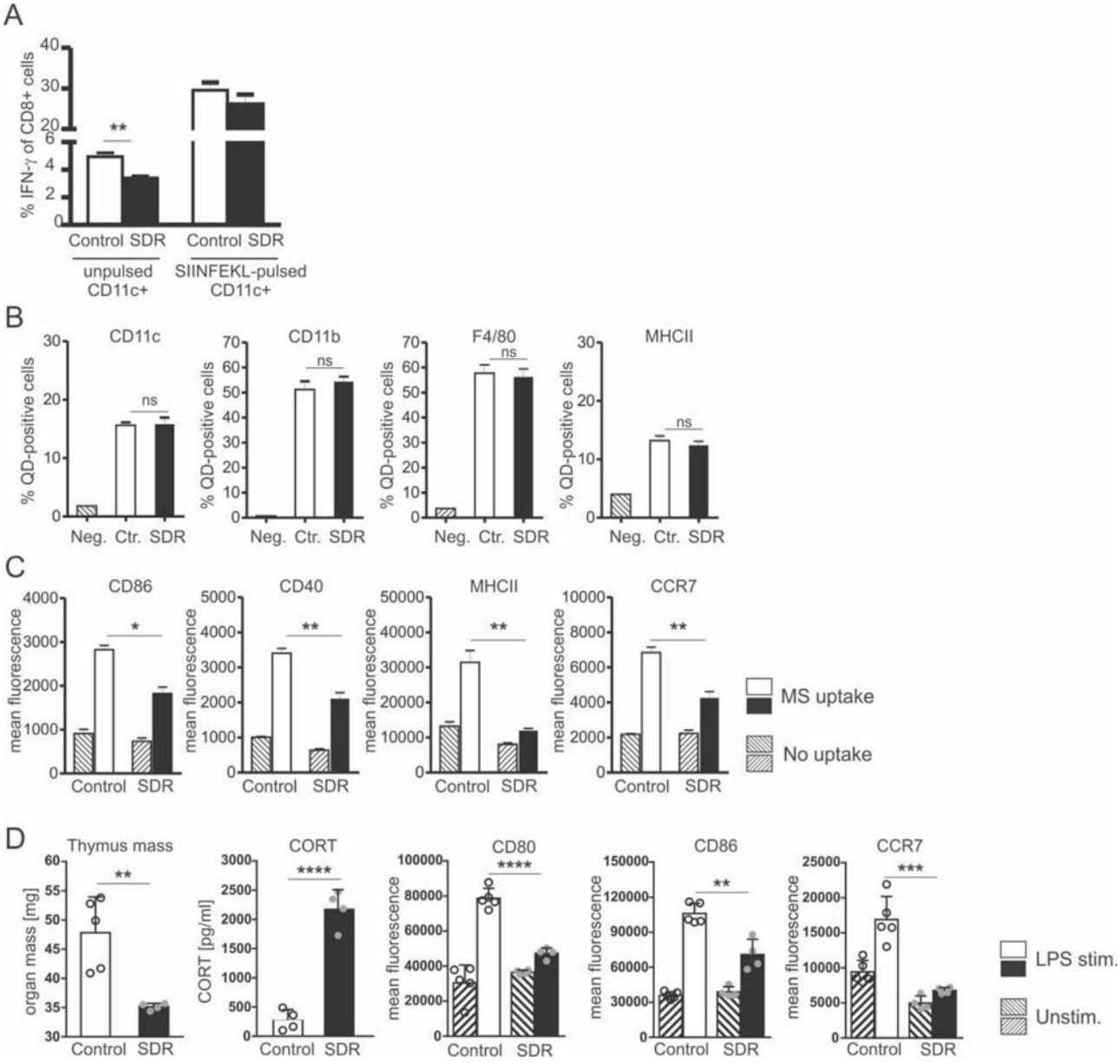

Fig. 4. DCs from SDR mice show an impaired T cell stimulatory function due to poor functional maturation. (A) An antigen presentation assay was performed using purified CD11c$^+$ DCs from spleens of SDR and control mice. These cells were incubated with PLGA-MS-OVA/CpG and MS polyI:C overnight, then pulsed with SIINFEKL-peptide (SIINFEKL-pulsed) or left unpulsed followed by 6 h of co-incubation with a SIINFEKL-specific CTL line. Activation of peptide-specific CTLs was determined by ICS IFN-γ and flow cytometry. Bar graphs represent the mean percentage of IFN-γ -producing SIINFEKL-specific CTLs (±SEM). Control, $n = 5$; SDR, $n = 5$. (B) Internalization of PLGA-MS by control and SDR splenocytes incubated at 4 °C (neg) or 37 °C with fluorescent Quantum dot (QD) positive PLGA-MS for 5 h. Cells were stained for CD11b, CD11c, F4/80 or MHC class II, respectively, and the percentage of QD-positive cells assessed by flow cytometry. All values represent the mean percentage of QD-positive cells (±SEM) of the respective cell type. Control, $n = 5$; SDR, $n = 5$. (C) Maturation of CD11c$^+$ cells upon PLGA-MS uptake *in vitro*. Splenocytes of mice were incubated with fluorescent QD-positive PLGA-MS overnight. After 18 h, QD-positive cells were assessed for surface expression of CD86, CD40, MHC class II, and CCR7 by flow cytometry and compared to the expression on QD-negative cells (no uptake, dashed bars), respectively. All values represent the mean fluorescence for the respective surface markers (±SEM) of QD-positive cells. Control, $n = 5$; SDR, $n = 5$. (D). Thymus mass and plasma CORT level of individual SDR and control mice was assessed and correlated with splenic CD11c + maturation after *in vitro* stimulation with LPS overnight. All values represent the mean fluorescence for CD86, CD80, and CCR7 surface expression (±SEM) on CD11c + cells of LPS-stimulated splenocytes compared to non-stimulated splenocytes (dashed bars. Control, $n = 5$; SDR, $n = 4$. Shown are representative results from two independent experiments. Significance is indicated with $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

responsiveness. Importantly, DCs from stressed mice were also found to exhibit a significantly lower expression of CCR7, which plays a pivotal role in directing activated DCs from the periphery to the T cell areas of draining lymph nodes (Pizzurro and Barrio, 2015; De Vries et al., 2003). In agreement with this notion, we found DCs from stressed mice had a significantly impaired capacity to migrate from the peripheral injection site to the regional LNs (Fig. 5A). The decreased trafficking and accumulation of Ag loaded DCs in the dLNs was associated with a deficiency in priming a SIINFEKL specific CTL response in congenic OT I hosts (Fig. 3B), suggesting that peripheral migratory DCs in stressed mice fail to initiate vaccine specific T_{CD8+} responses due to a low migratory capacity. This conclusion is consistent with both preclinical and clinical studies showing that the magnitude of the T cell response

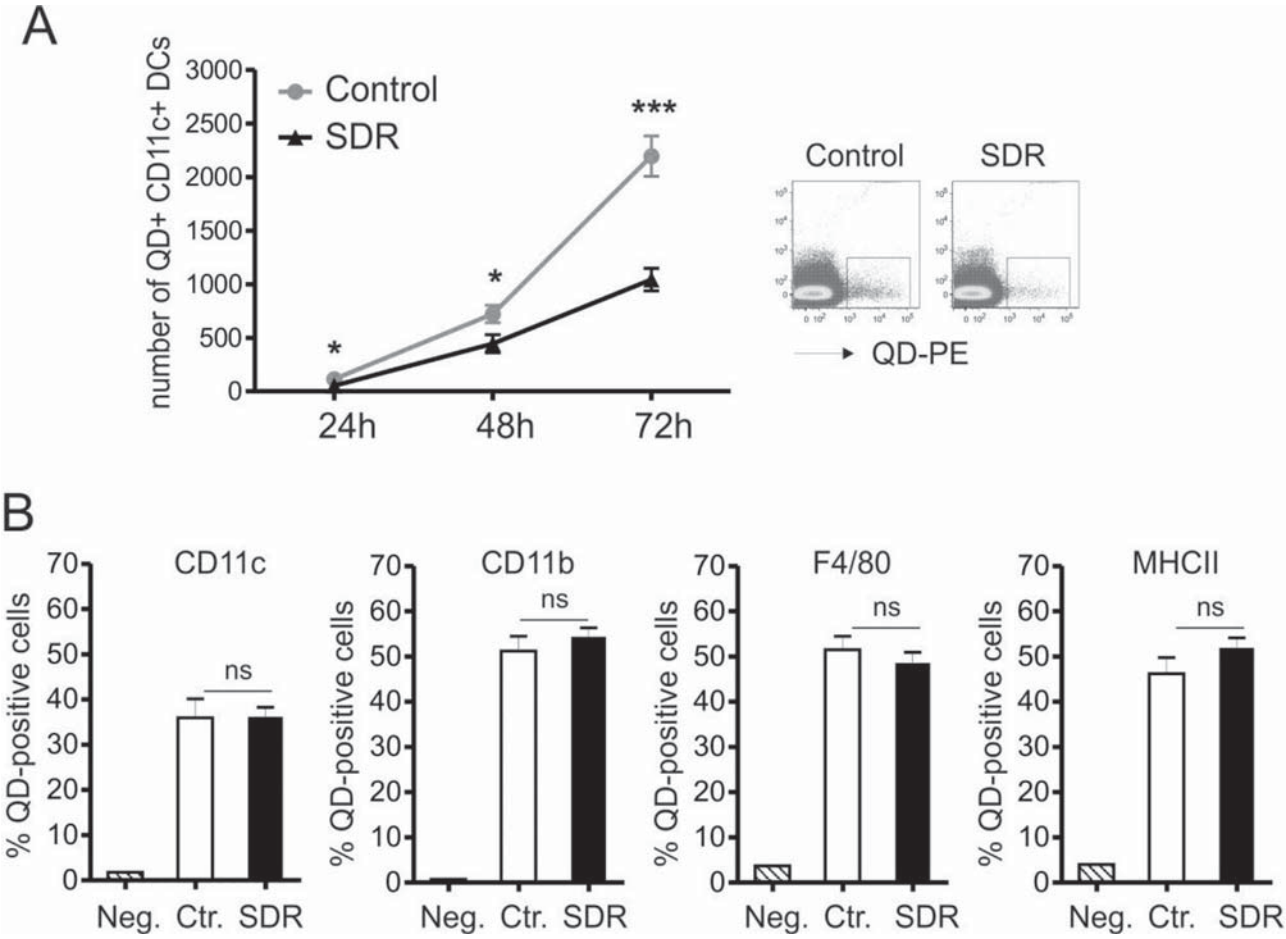

Fig. 5. Influence of chronic stress on the migration of CD11c$^+$ DCs to draining lymph nodes. (A) The ability of CD11c$^+$ cells to migrate from the site of s.c. PLGA-MS injection into the draining LN was analyzed. C57BL/6 mice received injections of fluorescent quantum dot (QD)-containing PLGA-MS into both hind footpads. 24 h (Control, $n = 4$; SDR, $n = 5$), 48 h (Control, $n = 9$; SDR, $n = 6$) and 72 h (Control, $n = 13$; SDR, $n = 12$) after PLGA-MS injection, both popliteal LNs were harvested, pooled and analyzed for QD-positive, CD11c$^+$ cells by flow cytometry. All values represent the total number of recovered QD-positive CD11c$^+$ cells (±SEM) detected within dLNs of treated mice. (B) For the analysis of PLGA-MS uptake *in vivo*, mice received injections of QD-positive PLGA-MS into both hind footpads. 5 h after PLGA-MS injection, single cell suspensions of the feet were surface stained for CD11c, CD11b, F4/80, or MHC class II and the percentage of QD-positive cells were assessed by flow cytometry. All values represent the mean percentage of QD-positive cells of the respective cell type (±SEM). Control, $n = 5$; SDR, $n = 5$ and are representative results from two independent experiments. Significance is indicated with $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

correlates with the ability of DCs to migrate to lymph nodes and thus with their maturation state (Sabado and Bhardwaj, 2015; Mitchell et al., 2015).

Non migratory lymphoid resident DCs also play an important role in the priming of T_{CD8+} cells through the transfer of the antigen from immigrating professional APCs (e.g. DCs and macrophages) and presentation through the MHC class I pathway. We found that isolated lymphoid resident DCs from SDR mice were also compro mised in their ability to functionally mature upon PLGA MS uptake (Fig. 4C) and were significantly less efficient in priming OVA specific CTLs *in vitro* (Fig. 4A). Hence impaired cross priming via LN resident DCs likely contributes to the impaired induction of the T_{CD8+} cell response in stressed mice. Collectively, our data pro vide evidence that intrinsic DC dysfunction in chronically stressed mice directly impairs the priming of T_{CD8+} responses *in vivo*, most likely as a result of inefficient migration of peripheral migratory DCs, but also possibly influenced by suboptimal MHC antigen pre sentation by lymphoid resident DCs (Fig. 3D).

Although alterations in DC functions in cancer patients were described almost two decades ago, DC dysfunction and the cellular mechanisms of abnormal differentiation and migration are still not well characterized. An understanding of how host factors regulate DCs and how this impacts the efficiency of DC based cancer vacci nes and other immunotherapeutic approaches is far from complete and many current clinical trials are focusing on critical parameters to improve DC function. Here, we describe for the first time that chronic psychological stress suppresses the functional activity of DCs in the context of anti cancer vaccines by impeding their mat uration and migration, thereby suppressing subsequent anti tumor specific T_{CD8+} responses. The implications of this observation are potentially far reaching: It is estimated that 20 to 47% of oncology patients worldwide experience severe chronic stress, with fre quency and severity increasing with advanced stages of illness (Thornton et al., 2007; Mehnert et al., 2014).

For cancer patients such a stress related dysfunction of DCs could result in marked deficiency in the induction of anti tumor immunity and low responses to therapies that require endogenous DCs to process and present tumor Ag to T cells. As patients DCs are being targeted in multiple immunotherapeutic clinical trials worldwide it is of high importance to fully understand the impact of psychological stress on DC functionality in therapeutic settings. We are confident that the potential of cancer vaccines will be more fully realized as we gain an advanced understanding of the mech anisms that limit the efficiency of anti cancer vaccines, ultimately enabling the development of immunotherapeutic strategies which overcome these limitations.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed

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