The Immunomodulatory Role of Endogenous Glucocorticoids in Ovarian Cancer

Die immunmodulatorische Bedeutung der lokalen Aktivierung von endogenem Cortison im Ovarialkarzinom

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Affidavit

I hereby declare that my thesis entitled "The immunomodulatory role of endogenous glucocorticoids in ovarian cancer" is the result of my own work. I did not receive any help or support from commercial consultants or others. All sources and/or materials applied are listed and specified in the thesis. Furthermore, I verify that this thesis has not been submitted as part of another examination process, neither in identical nor in similar form.

Würzburg, September 2012 Ahmed Adel Seida

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0.1 Summary

Ovarian cancer currently causes ~6,000 deaths per year in Germany alone. Since only palliative treatment is available for ovarian carcinomas that have developed resistance against platinum-based chemotherapy and paclitaxel, there is a pressing medical need for the development of new therapeutic approaches. As survival is strongly influenced by immunological parameters, immunotherapeutic strategies appear promising. The research of our group thus aims at overcoming tumour immune escape by counteracting immunosuppressive mechanisms in the tumour microenvironment.

In this context, we found that tumour-infiltrating myeloid-derived suppressor cells (MDSC) or tumour associated macrophages (TAM) which are abundant in ovarian cancer express high levels of the enzyme 11β-hydroxysteroid dehydrogenase1 (11β-HSD1). This oxido-reductase enzyme is essential for the conversion of biologically inactive cortisone into active cortisol. In line with this observation, high endogenous cortisol levels could be detected in serum, ascitic fluid and tumour exudates from ovarian cancer patients. Considering that cortisol exerts strong anti-inflammatory and immunosuppressive effects on immune cells, it appears likely that high endogenous cortisol levels contribute to immune escape in ovarian cancer. We thus hypothesised that local activation of endogenous glucocorticoids could suppress beneficial immune responses in the tumour microenvironment and thereby prevent a successful immunotherapy. To investigate the *in vivo* relevance of this postulated immune escape mechanism, irradiated PTEN$^{loxP/loxP}$ loxP-*Stop*-loxP-kras^{G12D} mice were reconstituted with hematopoietic stem cells from either glucocorticoid receptor (GR) expressing mice (GR$^{loxP/loxP}$) or from mice with a T cell-specific glucocorticoid receptor knock-out (lck-Cre GR$^{loxP/loxP}$) mice. In the host mice, the combination of a conditional PTEN knock-out with a latent oncogenic kras leads to tumour development when a Cre-encoding adenovirus is injected into the ovarian bursa. Using this model, mice that had been reconstituted with GC-insensitive T cells showed better intratumoural T cell infiltration than control mice that had received functionally unaltered GR$^{loxP/loxP}$ cells via adoptive transfer. However, tumour-infiltrating T cells mostly assumed a Foxp3^{+} (regulatory) phenotype and survival was even shortened in mice with cortisol-insensitive T cells. Thus, endogenous cortisol seems to inhibit immune cell infiltration in ovarian cancer, but productive anti-tumour immune responses might still be prevented by further factors from the tumour microenvironment.

Thus, our data did not provide a sufficiently strong rationale to further pursue the antagonisation of glucocorticoid signalling in ovarian cancer patients, Moreover, glucocorticoids are frequently administered to cancer patients to reduce inflammation and swelling and to prevent chemotherapy-related toxic side effects like nausea or hypersensitivity reactions associated with paclitaxel therapy. Thus, we decided to address the question whether specific signalling pathways in innate immune cells, preferentially in NK cells, could still be activated even in the presence of GC. A careful investigation of the various activating NK cell receptors (i.e. NKp30, NKp44, NKp46), DNAM-1 and NKG2D) was thus performed which revealed that NKp30, NKp44 and NKG2D are all down-regulated by cortisol whereas NKp46 is actually induced by cortisol.

Interestingly, NKp46 is the only known receptor that is strictly confined to NK cells. Its activation via crosslinking leads to cytokine release and activation of cytotoxic activity. Stimulation of NK cells via NKp46 may contribute to immune-mediated tumour destruction by triggering the lysis of tumour cells and by altering the cytokine pattern in the tumour microenvironment, thereby generating more favourable conditions for the recruitment of antigen-specific immune cells. Accordingly, our observation that even cortisol-treated NK cells can still be activated via NKp46 and CD2 might become valuable for the design of immunotherapies that can still be applied in the presence of endogenous or therapeutically administered glucocorticoids.

0.2 Zusammenfassung

Ovarialkarzinome verursachen allein in Deutschland jährlich ca. 6.000 Todesfälle. Da bei Ovarialkarzinomen, die eine Resistenz gegen eine platinbasierte Chemotherapie mit cis-Platin und gegen Paclitaxel entwickelt haben, nur eine palliative Behandlung möglich ist, gibt esbesteht ein dringenden dringender Bedarf an der Entwicklung von neuen Therapieansätzen. Das Überleben der Patientinnen ist sehr stark von immunologischen Parametern beeinflusst, und somit erscheinensodass immuntherapeutische Strategien als ein vielversprechender Ansatzerscheinen. Das Ziel der Forschung in unserer Gruppe ist daher, dem „Tumor-Immun-Escape“ durch eine Verhinderung von immunosuppressiven Mechanismen in im der Tumormikromilieu-Mikroumgebung entgegenzuwirken.

In diesem Zusammenhang haben wir gefundenentdeckt, dass Tumor-infiltrierende Suppressorzellen der myeloiden Ursprungsschen Reihe (MDSC) oder Tumor-assoziierte Makrophagen (TAM), die in Ovarialkarzinomen reichlich vorhanden sind, das Enzym 11β-Hydroxysteroid-Dehydrogenase 1 (11β-HSD1) in großer Menge exprimieren. Diese Oxido-Reduktase ist essentiell bei derfür die Umwandlung von biologisch inaktiven Cortison in biologisch aktives Cortisol. In Übereinstimmung damit, werden hohe endogene Cortisol Spiegel in Seren, Azites und Tumorsekreten von Ovarialkarzinom-Patientinnen gemessen. Unter Berücksichtigung der starken anti-inflammatorischen und immunsuppressiven Eigenschaften von Cortisol, erscheint es sehr wahrscheinlich, dass ein hoher endogener Cortisol-Spiegel zum „Immune-Escape“ von Ovarialkarzinomzellen beiträgt. Unsere Vermutung ist Wir stellten daher die Hypothese auf, dass die lokale Aktivierung von endogenen Glucocorticoiden zu einer Unterdrückung der nützlichen Immunantwort in der Tumor-Mikroumgebung führt und eine erfolgreiche Immuntherapie verhindert.

Um die in vivo Relevanz dieses postulierten „Immune-Escape“ Mechanismuses zu untersuchen, wurden bestrahlte $PTEN^{loxP/loxP}$ loxP-Stop-loxP-$kras^{G12D}$ Mäuse mit Hämatopoetischen hämatopoietischen Stammzellen entweder aus Glucocoprticoid-Rezeptor (GR) exprimierenden Mäusen ($GR^{loxP/loxP}$) oder aus Mäusen mit einem T-Zell spezifischen Glucocoprticoid-Rezeptor knock-out (lck-Cre $GR^{loxP/loxP}$) rekonstituiert. In diesen Mäusen führte die Kombination von konditionellem PTEN knock-out mit einer latenten Expression von oncogenen onkogenen Kras zur Tumorentwicklung sobald ein für Cre-Rekombinase codierendes Adenovirus in die Ovarien-ovarielle Bursa injiziert wurdewird. Mit Hilfe von

diesesm Modells wurde gezeigt, dass Mäuse, die mit GC-unempfindlichen T-Zellen rekonstituiert worden waren eine bessere intratumorale T-Zell Infiltration zeigten im Vergleich zu Kontroll-Mäusen, die über einen adaptiven Transfer funktionell unveränderte $GR^{loxP/loxP}$ Zellen erhalten hatten. Tumor-infiltrierende T-Zellen haben aber in der Mehrzahl einen hauptsächlich angenommen Foxp3+ (regulatorischen) Phänotyp angenommen, sodass und das Überleben dieser Zellen war sogar verkürzt in Mäusen mit Cortisol-unempfindlichen T-Zellen sogar eine verkürzte Überlebenszeit aufwiesen. Somit scheint endogenes Cortisol die Infiltration von Immunzellen beim Ovarialkarzinom zu inhibieren, aber eine produktive antitumorale Immunantwort könnte scheint trotzdem verhindert werden durch andere Faktoren aus im Tumormikromilieu verhindert zu werden.der Tumor-Mikroumgebung.

Somit bieten unsere Daten keine ausreichend starke Begründung, für eineum das Konzept der Antagonisierung endogener,der durch Glucocorticoide ausgelösten ausgelöster Signale in Ovarialkarzinom-Patientinnen weiter zu verfolgen. Des Weiteren werden Glucocorticoide oft Krebspatienten verabreicht, um Entzündungen und Schwellungen zu reduzieren sowie Chemotherapie. bedingte Nebeneffekte wie Übelkeit oder Überempfindlichkeitsreaktionen, die mit einer Paclitaxel-Therapy einhergehen, zu verhindern. Daher wurde der Frage nachgegangen, ob spezifische Signalwege im angeborenem Immunsystem, insbesondere in NK-Zellen, auch in Anwesenheit von GC noch aktiviert werden können. Eine Untersuchung der verschiedenen NK-Zellen aktivierenden Rezeptoren (NKp30, NKp44, NKp46, DNAM-1 und NKG2D) wurde durchgeführt. Dabei wurde eine Herunterregulation von NKp30, NKp44 und NKG2D sowie eine Induktion von NKp46 durch Cortisol festgestellt.

Von besonderem Interesse ist, dass NKp46 der einzige bekannte Rezeptor ist, der nur von NK-Zellen exprimiert wird. Seine Aktivierung bewirkt eine Cytokinfreisetzung Zytokinfreisetzung und eine Aktivierung Induktion der zytotoxischen AktivitätNK Zell-Antwort. Eine Stimulation der NK-Zellen über NKp46 könnte zur immun-vermittelten Tumorzerstörung durch Auslösen der Tumorzell-Lyse und durch eine Veränderung des Cytokinexpressionsmusters Zytokinexpressionsmusters in im Tumormikromilieu der Tumor-Mikroumgebung beitragen. Somit würden verbesserte Bedingungen für die Rekrutierung von antigen-spezifischen Immunzellen generiert. Unsere Beobachtung, dass selbst Cortisol behandelte NK-Zellen immer noch über NKp46 und CD2 aktiviert werden können, könnten nützlich zur Entwicklung von Immuntherapien sein, die in Gegenwart von endogenen oder therapeutisch verabreichten Glucocorticoide angewendet werden sollen.

1 Introduction

1.1 Ovarian cancer

1.1.1 Epidemiology, diagnosis, classification, prognosis, therapy

Ovarian carcinoma is one of the leading causes of death from gynecological malignancies in the western world (Fehrmann et al., 2007). Internationally; ovarian cancer is the sixth most common cancer among women (Riman et al., 1998).

Ovarian cancer is the 2nd most common violent affection of the female sexual organs, and it is mostly diagnosed in women aged >40 years. Due to the lack of cancer screening tests, ovarian carcinomas are frequently diagnosed at late stages. In Germany 9000 (Europe 66,000) new ovarian cancer cases and 5,500 (Europe 40,000) deaths were registered in 2008 (http://www.oncgnostics.com).

The causes of ovarian cancer are poorly understood. Ovarian cancer is thought to be consequence of result from an accumulation of genetic alterations (Aunoble B et al., 2000; Fehrmann et al., 2007). Over 90% of ovarian cancers occur sporadically. Ovulation appears to increase the danger as does family history, increasing age, infertility, and usage of ovulatory drugs. Pregnancy and the use of oral contraceptives, as well as possibly breast-feeding, lower the risk. Furthermore, tubal ligation or hysterectomy with ovarian preservation also lowers the risk (Herbst, 1994). The median age of patients with ovarian cancer is 60 years, and the average life span risk for women is about 1 in 70. A strong family history of ovarian or breast cancer is the major risk factor, while genetic predisposition is present in about 5 % of affected women (Cannistra, 2004).

Epithelial ovarian tumours, germ cell tumours and sex cord/stromal tumours are the main types of ovarian tumours. Epithelial ovarian tumours which constitute 80–90% of ovarian malignancies resulting are (according to recent findings) mostly derived from the surface epithelium of the fimbriated end of the fallopian tube (Dietl & Wischhusen, 2011) and constitute 80–90% of ovarian malignancies. Approximately, 15% of the epithelial tumours are borderline malignant while the rest are invasive cancers. There are several histopathological subgroups of epithelial ovarian cancers (EOC) (Riman et al., 1998). It has been reported that the EOC were categorized as follows: serous adenocarcinoma (43%), mucinous

adenocarcinoma (15%), endometroid adenocarcinoma (22%), clear cell adenocarcinoma (5%) and mixed or undifferentiated tumours (14%) (Booth et al., 1989; Riman et al., 1998).

The early discovery of ovarian cancer considerably decreases the mortality rate of ovarian cancer patients. For early detection of ovarian cancer, six markers (leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor, and CA-125) are utilized to distinguish healthy versus cancer patients. The use of six markers showed great diagnostic improvement (sensitivity 84%-98% at specificity 95%) over the use of only CA-125 alone (sensitivity 72% at specificity 95%) (Visintin et al., 2008).

The majority of cases are diagnosed at an advanced stage and the prognosis is poor, with 5-year survival rates less than 40%. Yearly, more than 100.000 women are estimated to die from the ovarian cancer (Parkin et al., 1993; Riman et al., 1998). Some classical clinic-pathological factors, such as age, stage, residual tumour after first laparotomy, differentiation grade, histiotype, and response to chemotherapy are vital prognostic markers for ovarian cancer (du Bois A et al., 2005; Fehrmann et al., 2007). Also, microarray technology at the present permits analysis of expression levels of thousands of genes and is broadly used to identify prognostic gene-expression profiles for all sorts of cancer (Fehrmann et al., 2007).

Generally, the first treatment for ovarian cancer is surgery which will consist of removal of the uterus, tubes and ovaries as well as any large nodules of cancer (http://www.gyncancer.com/ovarian-cancer.html). Furthermore, due to a possible contribution of pelvic and paraaortic node in ovarian carcinoma metastasis a bilateral lymphadenectomy is an important step in the surgical treatment of EOC and has a diagnostic, prognostic and therapeutic value (Morice et al., 2003).

Most patients will be left with residual disease and will therefore require postoperative chemotherapy after surgical removal of ovarian cancer (Berkenblit and Cannistra, 2005). Platinum-based chemotherapy (cisplatin or carboplatin) is a practical primary step for the chemotherapy (Harries and Kaye, 2001; Berkenblit and Cannistra, 2005). Also, it has been reported that addition of taxanes to platinum-containing regimens brings a significant benefit and the standard of care has become the combination of carboplatin and paclitaxel (Harries and Kaye, 2001; Berkenblit and Cannistra, 2005). Still, the majority of patients with late stage ovarian cancer will relapse and will be candidates for further chemotherapy, which can

palliate symptoms and improve survival rate even in recurrent disease (Harries and Kaye, 2001). Radiotherapy, in contrast, cannot be applied since ovarian carcinomas grow close to the extremely radio-sensitive gut that would be too severely affected by irradiation treatment.

1.1.2 Spontaneous mouse model for ovarian cancer (Dinulescu et al., 2006)

A Tyles Jacks and colleagues developed a spontaneous mouse model to recapitulate endometrioid ovarian cancer which is characterized by an inactivation of the tumor suppressor PTEN and a hyperactivation of kras used to investigate the functional immunomodulatory role of endogenous glucocorticoids in ovarian cancer. For this model, an adenovirally encoded Cre-recombinase (AdCre) was is injected directly into the ovarian bursa of 129 S4/SvJae-C57BL/6JloxP-Stop-loxP-K-ras$^{G12D/+}$Pten$^{loxP/loxP}$ mice to induce the deletion of the PTEN tumour suppressor gene and the activation of the latent oncogenic K-ras^{G12D} (Dinulescu et al., 2006). Due to the deletion of PTEN and the activation of K-ras, the mice developed ovarian cancer of the endometrioid subtype which becomes immunohistochemically detectable visible within 6-7 weeks. Later, the mice also developed peritoneal metastases as evidenced by the formation of ascites in the abdominal cavity.

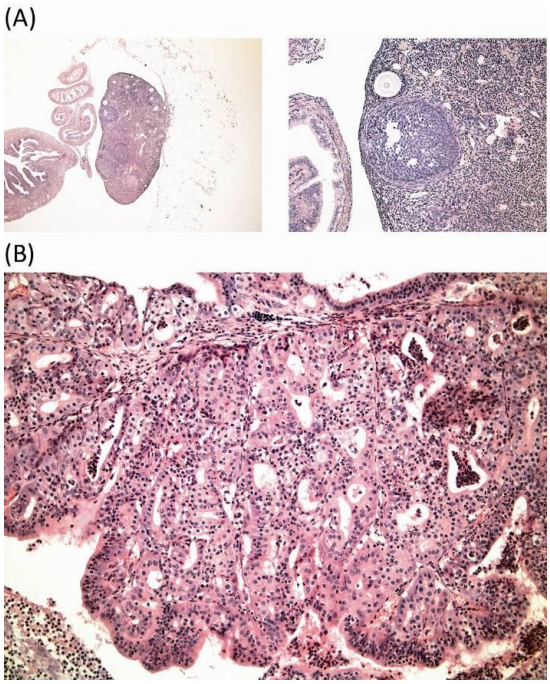


Figure (1): Spontaneous mouse model for ovarian cancer. Injection of adenoviral Cre-recombinase into the ovarian bursa induces the deletion of the PTEN tumour suppressor gene and the activation of the latent oncogenic K-ras. Mice thus developed an endometrioid ovarian carcinoma within 6-7 weeks (B). No cancer developed in contralateral non-treated ovaries (A). Experiment performed and photographed by Dr. Sebastian Häusler.

1.2 Adrenal Glucocorticoids

Glucocorticoids (GCs) (Cortisol and Corticosterone) are a group of steroid hormones that bind to the glucocorticoid receptor (GR), which is present in almost every vertebrate animal cell. The name GC (glucose + cortex + steroid) derived from their function in the regulation of the metabolism of glucose and from, their synthesis in the adrenal cortex.

Hydrocortisone (Cortisol) is the most vital human GC. It is necessary required to regulate or support a variety of vital cardiovascular, metabolic, immunological, and homeostatic functions. Additional to that, various synthetic GCs are obtainable such as dexamethasone; these are used either as alternative therapy in GC deficiency or to inhibit the immune system in autoimmune diseases and to stop ameliorate chemotherapy-related side effects.

By their mode of action, GCs bind to the cytosolic GR which then translocates to the nucleus where it binds to Glucocorticoid response elements (GRE) thereby leading to an up-regulation of anti-inflammatory protein expression (transactivation). In the cytosol, GCs can further repress the expression of pro-inflammatory proteins by preventing the translocation of other transcription factors from the cytosol into the nucleus (transrepression) (Rhen and Cidlowski, 2005).

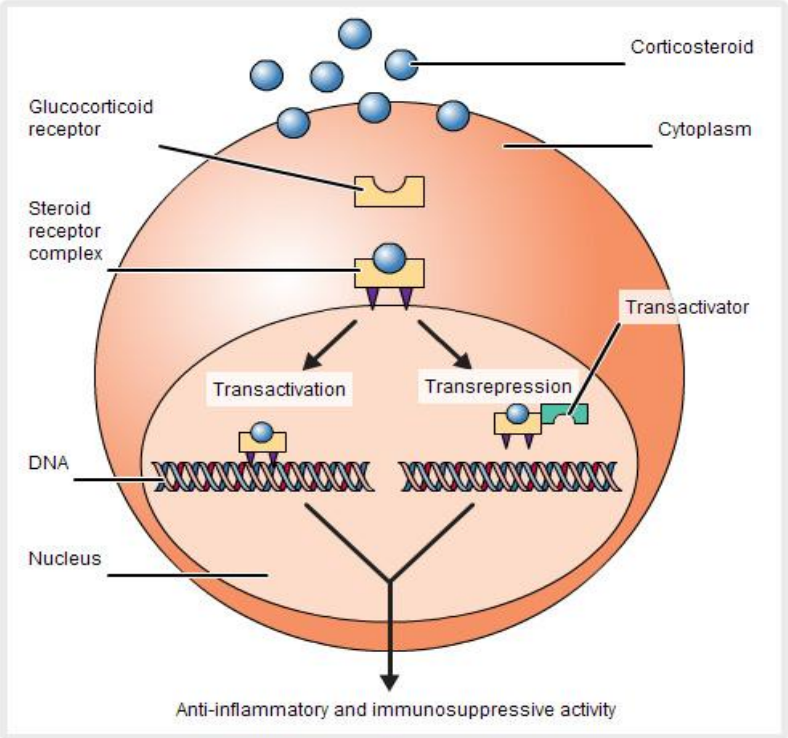


Figure (2): Mode of action of GCs (http://www.epgonline.org).

1.2.1 Origin of Adrenal Steroids

Adrenal steroids are a group of steroid hormones secreted and synthesized by the adrenal cortex. Adrenal steroids can be categorized as GCs (cortisol and corticosterone), mineralocorticoids (aldosterone), androgens dehydroepiandrosterone (DHEA) and androstenedione. All steroid hormones are derivatives of Cholesterol which require numerous enzymatic steps during the synthesis of adrenal steroids.

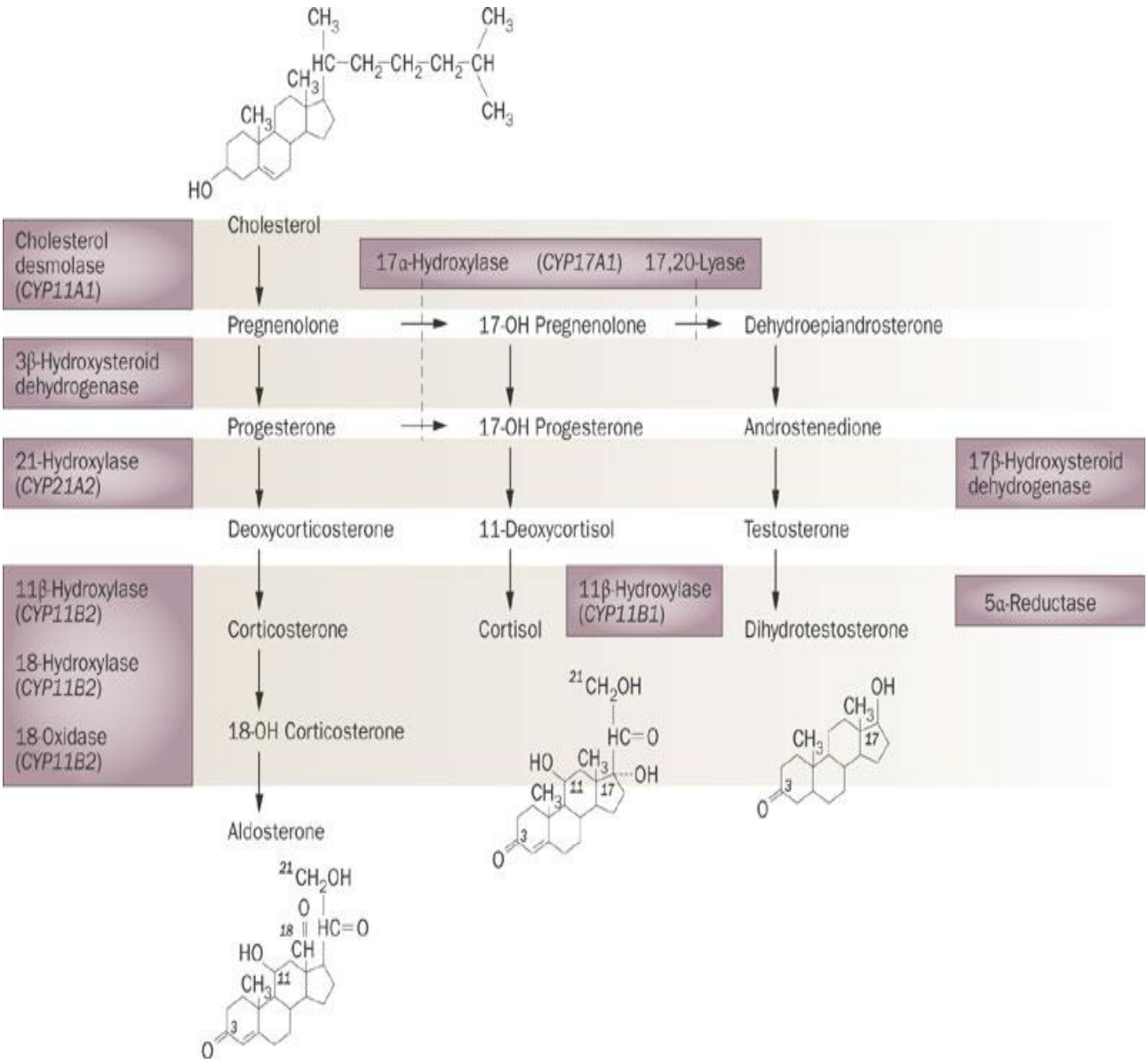


Figure (3): Pathways of steroid biosynthesis in the adrenal cortex (White, 2009).

1.2.1.1 Systemic control of cortisol secretion regulated by the hypothalamic-pituitary-adrenal axis

Systemically, secretion of adrenal GC is coordinated by the hypothalamus-pituitary-adrenal axis. Neuroendocrine stimuli delivered to hypothalamus which govern GCs release are stress (Tasker et al., 2006) and pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) (Turnbull and Rivier, 1999). In addition, the circadian rhythm generator (Abe et al. 1979; Vrang et al. 1995) and a negative feedback loop (Tasker et al., 2006) influence the availability of cortisol. About 75% of the cortisol in circulation is bound to a plasma protein called the corticosteroid binding globulin (CBG), while the rest is bound to serum albumin. Normally, cortisol in circulation is inactive, and only becomes biologically active when it becomes unbound from transcortin.

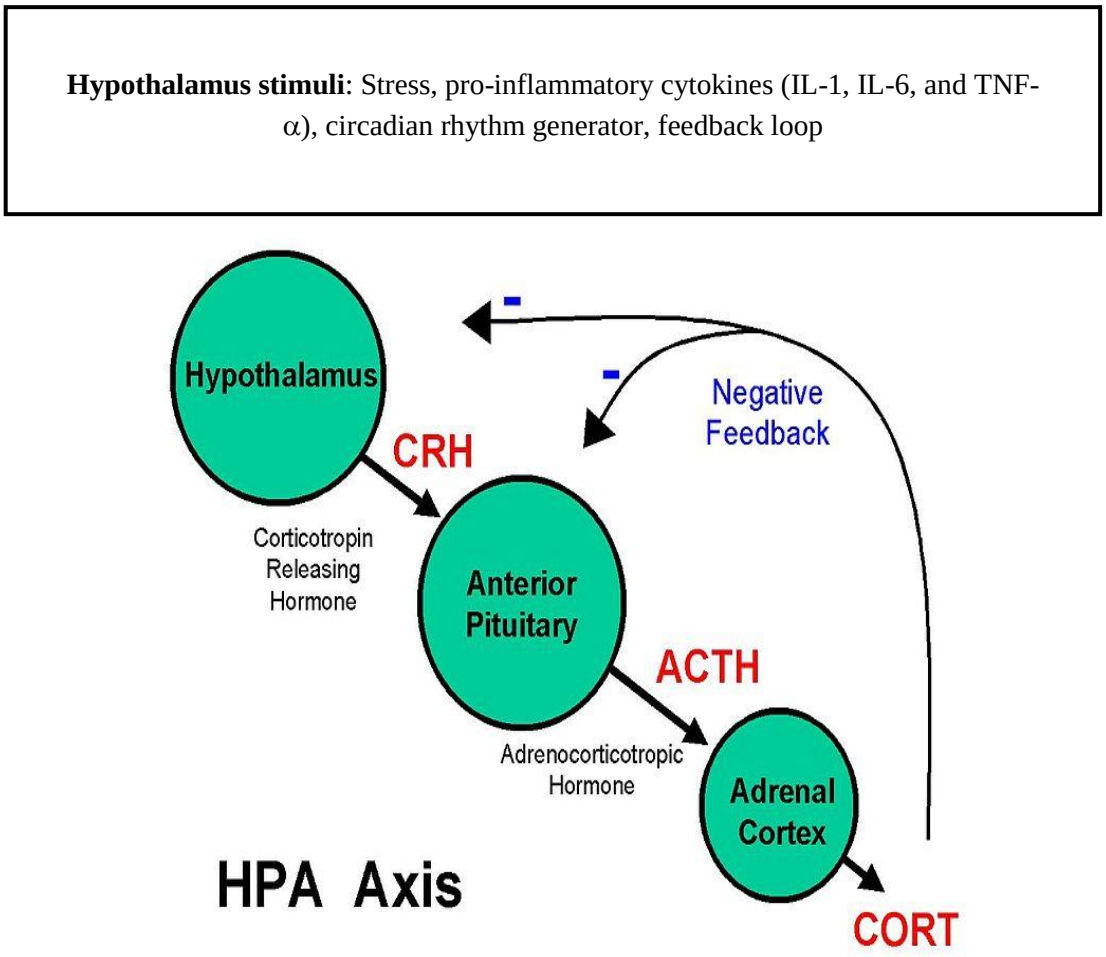


Figure (4): Systemic control of cortisol secretion regulated by the hypothalamic-pituitary-adrenal axis (Open access image from wikipedia website).

1.2.2 Local control and metabolism of GCs

The local regulation of GCs in the tissues is controlled by 11beta-Hydroxysteroid dehydrogenases (11β-HSD). The two iso-enzymes 11β-HSD1 and 11β-HSD2 catalyse the interconversion of hormonally active cortisol (F) and inactive cortisone (E). Both isoenzymes are localized in endoplasmic reticulum. The type 1 and type 2 11β-HSD isozymes share only 14% homology and are separate gene products with different physiological roles, different regulation, and different tissue allocation (Stewart and Krozowski, 1999; Vogt et al., 2002; Michael et al., 2003; Walker and Stewart, 2003). *In vivo*, 11β-HSD1 acts mostly as an oxidoreductase using NADP (H) as cofactor to generate cortisol. *In vitro*, however, its activity is bidirectional, acting as dehydrogenase (cortisol to cortisone) and reductase (cortisone to cortisol). 11β-HSD2, in contrast, acts exclusively unidirectional as NAD-dependent dehydrogenase, inactivating cortisol to cortisone (Walker and Stewart, 2003).

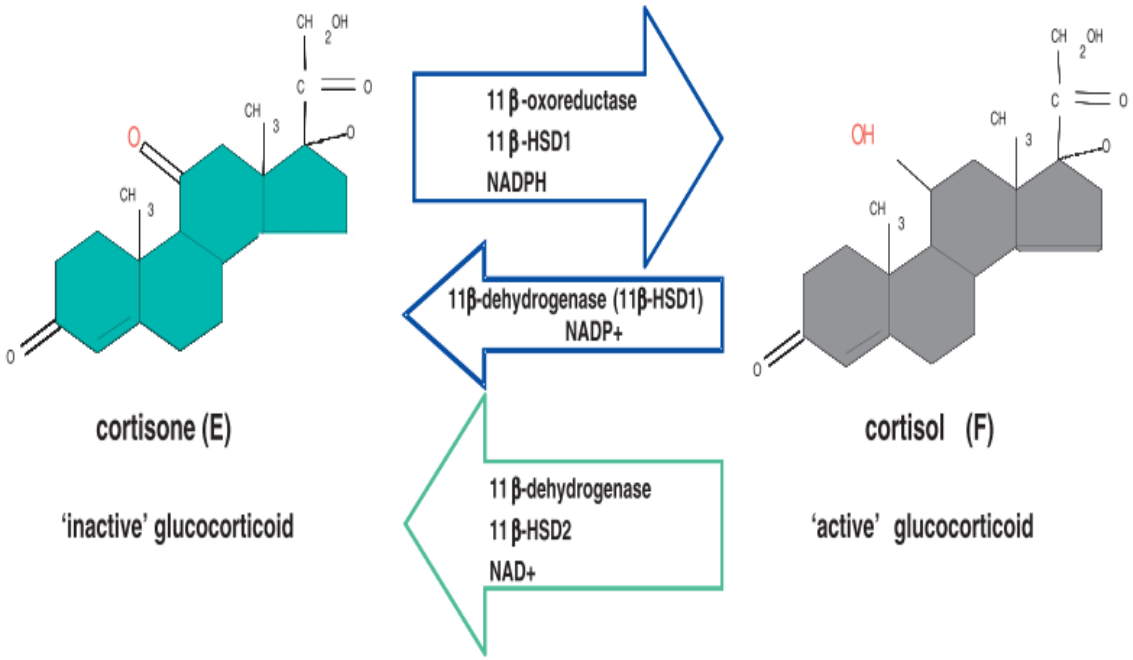


Figure (5): Local control and metabolism of GCs via 11β-HSD isozymes (Draper and Stewart, 2005).

Table (1): Comparison between 11β-HSD1 and 11β-HSD2 isozymes (Draper and Stewart, 2005).

	11β-HSD1	11β-HSD2
Chromosomal location	1q32·2	16q22
Size	Gene: 30 kb, 6 exons	6.2 kb, 5 exons
	Protein: 292 aa, 34 kDa	405 aa, 44 kDa
Enzyme family	Short chain dehydrogenase/reductase superfamily	Short chain dehydrogenase superfamily
Tissue expression	Liver, lung, gonads, pituitary, brain, adipose	Kidney, colon, salivary glands placenta
Enzyme kinetics	*In vitro* bidirectional	Only dehydrogenase
	In vivo mainly reductase	Cofactor NAD
	Cofactor NADP(H)	
Function	Supplies cortisol to GR	Protects MR from cortisol

1.2.2.1 Regulation of expression and activity of 11β-HSD1

11β-HSD1 expression and activity are regulated by different factors such as pro-inflammatory and anti-inflammatory cytokines, IL-1α, (Yong et al., 2002; Chapman et al., 2006), IL-1β (Escher et al., 1997; Cai et al., 2001; Tomlinson et al., 2001; Li et al., 2006; Chapman et al., 2006), TNF-α (Escher et al., 1997; Thieringer et al., 2001; Cai et al., 2001; Tomlinson et al., 2001; Chapman et al., 2006), IL-6 (Tomlinson et al., 2001; Friedberg et al., 2003), IL-4 and IL-13 (Thieringer et al., 2001; Hardy et al., 2006). Moreover, combined treatment with GCs (Cortisol or Dexamethasone) increases the expression of 11β-HSD1 (Tomlinson et al., 2002, Sun and Myatt, 2003; Li et al., 2006) and in combination with pro-inflammatory cytokines (TNF-α/IL-1β) synergistically increases expression and activity of 11β-HSD1 (Sun and

Myatt, 2003; Yang et al., 2009; Kaur et al., 2010; Ahasan et al., 2012). On the other hand, TNF-α/IL-1β down-regulated regulates the 11β-HSD2 expression (Cai et al., 2001; Cooper et al., 2001).

1.2.2.2 11β-HSD1and ovarian surface epithelium

Ovulation is a natural inflammatory event which includes haemodynamic, vascular, and biochemical changes leading to proteolytic breakdown of the follicle wall and release of an oocyte for fertilization (Espey, 1980; Hillier and Tetsuka 1998; Hillier, 2007). Many pieces of evidence indicate that the ovarian single-layered epithelium participates in repair of the ovarian surface after the 'wounding' caused by ovulation (Michael et al., 2003) though an involvement of epithelial cells from the fallopian tube has also been described (Dietl et al., 2011).Young et al showed that ovarian surface epithelial (OSE) cells express 11β-HSD1 and this expression can be up-regulated by pro-inflammatory cytokines such as IL-1 (Yong et al., 2002; Rae et al. 2004), this leads to a local anti-inflammatory environment since enhanced expression and thereby enhanced oxo-reductive activity of 11β-HSD1 increases the local availability of cortisol. This mechanism is thought to occur as a reaction to the ovulation-inducing LH surge (Yong et al., 2002) and is likely to contribute to the local resolution of inflammation during ovulation. In a pathological context, the pro- and anti-inflammatory processes involved in ovulation as injury-repair process may contribute to development of ovarian cancer, especially since many processes that are required for wound healing can also contribute to tumour growth (Schäfer and Werner, 2008). On the other hand, epidemiological data show that ovarian epithelial inflammation may play a role in ovarian carcinogenesis (Ness and Cottreau, 1999; Ness et al., 2000; Yong et al., 2002; Fleming, et al. 2006).

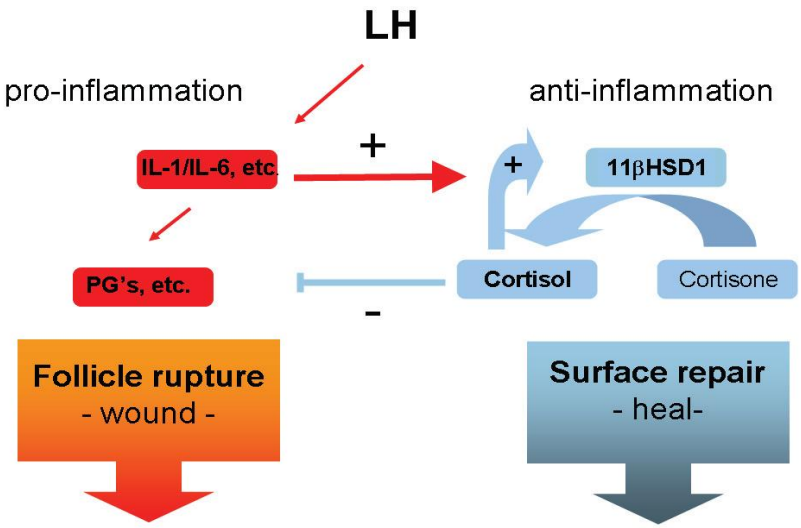


Figure (6): Role of 11β-HSD1 in the resolution of inflammation at the ovarian surface during ovulation. The ovulation-inducing LH surge induces an acute inflammatory response through increasing the local production of inflammatory cytokines (IL-1) and prostaglandins (PG). LH-induced IL-1 consecutively up-regulates the expression of 11β-HSD1 in granulosa cells and OSE cells, helping to increase the metabolism of inactive cortisone (E) into active anti-inflammatory cortisol (F) (Yong et al., 2002; Kenneth Scott Fegan, 2009).

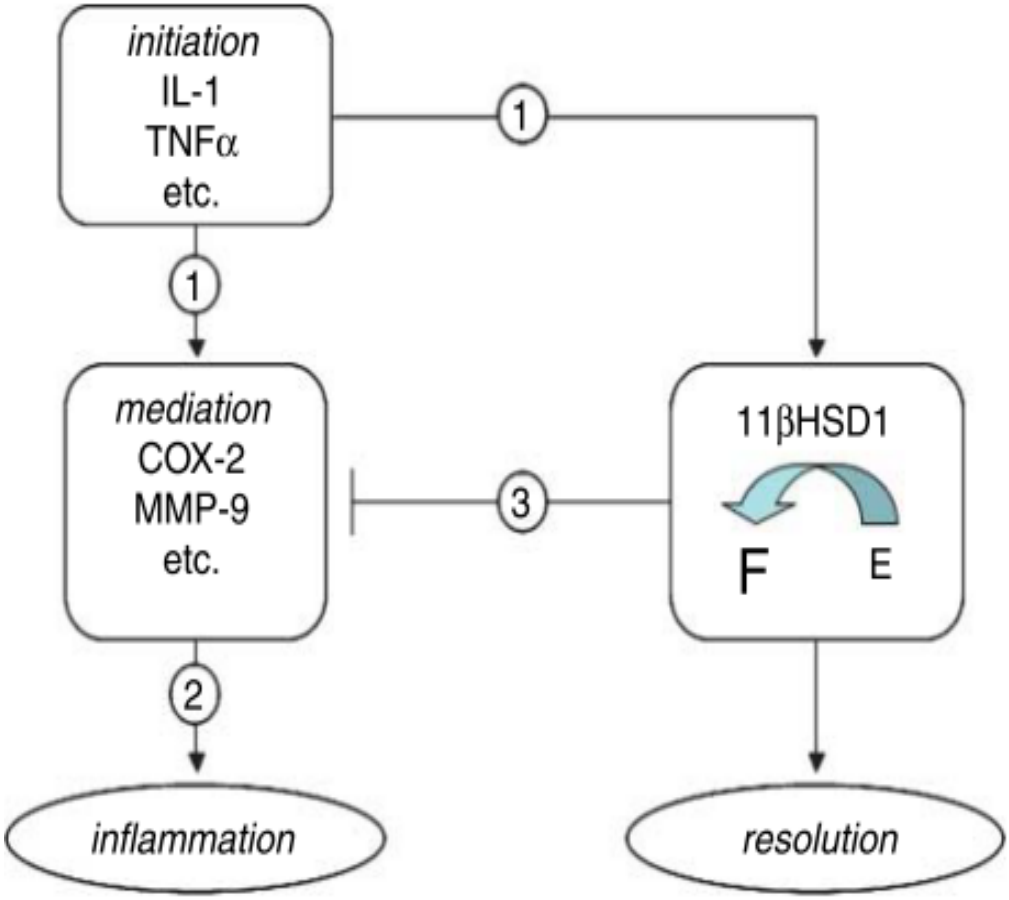


Figure (7): Intracrine amplification of anti-inflammatory GC action by 11β-HSD1. (1) Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α) increase the expression of inflammatory mediators, such as cyclo-oxygenase-2 (COX-2) and matrix metalloproteinase-9 (MMP-9). The inflammatory response includes an associated increase in 11β-HSD1 (and/or reduced 11β-HSD2) expression on mRNA level and enhances 11-oxoreductase enzymic activity. (2) Cytokine-induced inflammatory mediators bring about haemodynamic, vascular and biochemical changes associated with inflammation. (3) Active cortisol (F) formation from inactive cortisone (E) is locally increased due to cytokine-enhanced 11-oxoreductase activity of 11β-HSD1. Increased binding of cortisol (F) to the glucocorticoid receptor (GR) activates GR-mediated post-receptor anti-inflammatory signalling leading to resolution of inflammation (Tetsuka et al. 1999; Yong et al. 2002; Rae et al. 2004; Hillier, 2007).

1.2.2.3 11β-HSD1 expression in immune cells

11β-HSD1 has a role in the regulation of energy metabolism and the immune system by locally activating GCs (Nashev et al., 2007). Thieringer et al. showed that 11β-HSD1 is not constitutively expressed in human monocytes, but rather induced in human monocytes upon differentiation to macrophages. Further induction is possible in response to the cytokines IL-4 and IL-13 (Thieringer et al., 2001). On the other hand, human monocyte-derived DCs are able to activate cortisone to cortisol as a consequence of up-regulated expression of the enzyme 11β-HSD1 (Freeman et al., 2005). In mice, a single injection of thioglycolate into the peritoneum was sufficient to elicit an induction of 11β-HSD1 activity in macrophages, which then increased intracellular levels of active GCs, thereby promoting macrophage phagocytosis of leukocytes undergoing apoptosis (Gilmour et al., 2006). Moreover, Zhang et al demonstrated that 11β-HSD1 transcripts, protein, and enzyme activities are found in murine $CD4^+$, $CD8^+$, and $B220^+$ lymphocytes, as well as $CD11c^+$ DCs (Zhang et al., 2005).

1.2.3 GCs effects on the immune cells

Regulation of GCs is essential for the maintenance of immune homeostasis. GCs can be employed in situations to avoid excessive immunosuppression, death from overwhelming infection, or death from cytokine storm induced shock (Sternberg, 2006). GCs can affect immune cells directly and indirectly via increasing amounts of anti-inflammatory cytokines and decreasing levels of pro-inflammatory cytokines. Moreover, GC can induce apoptosis in all immune cells and inhibit the immune response (Sternberg, 2006).

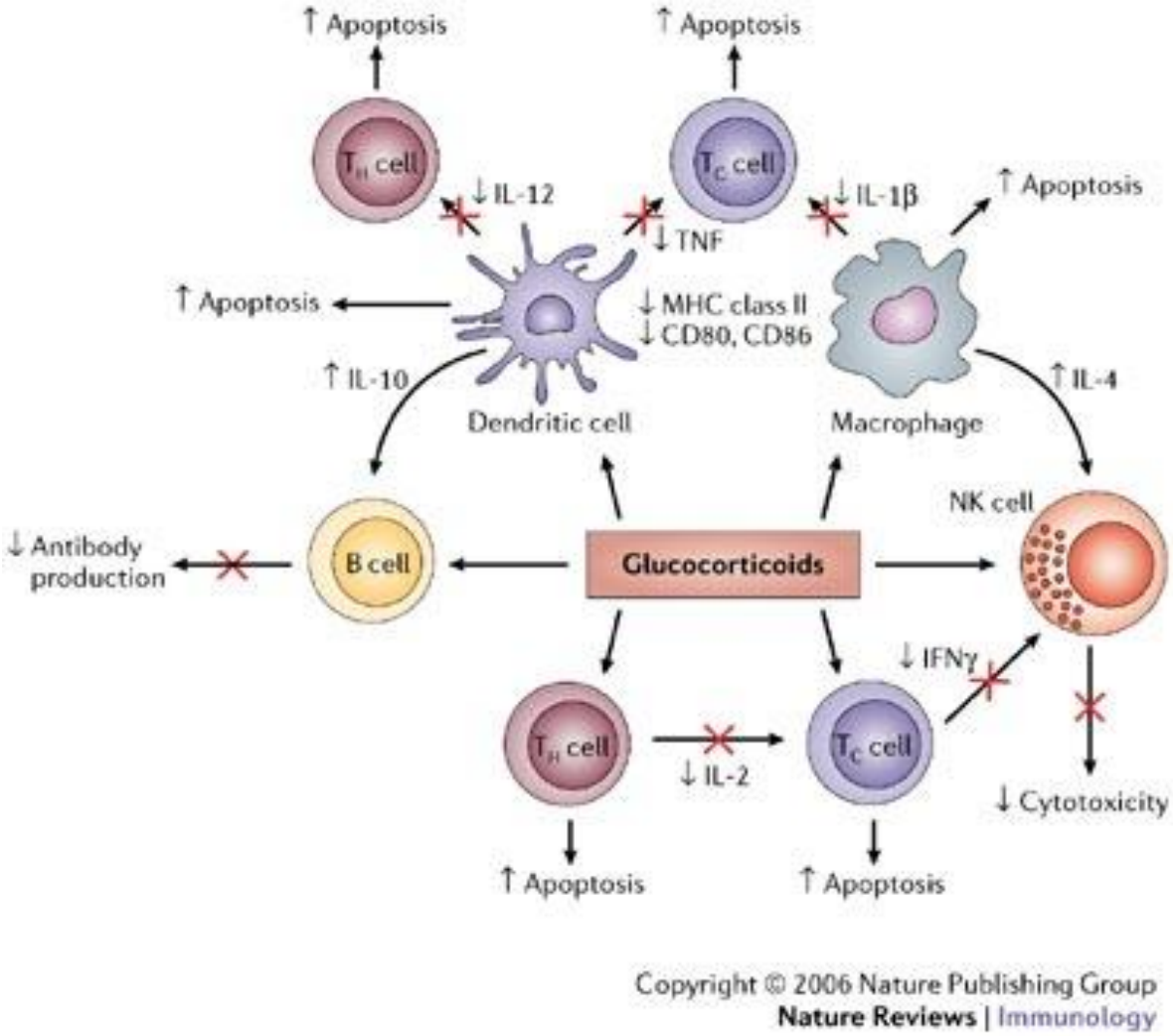


Figure (8): GCs effects on the immune cells. GCs act on immune cells both directly and indirectly to suppress the induction of pro-inflammatory responses. They inhibit the production of pro-inflammatory cytokines, such as interleukin-1beta (IL-1β) and tumour-necrosis factor-alpha (TNF-α), while promoting the production of anti-inflammatory cytokines, such as IL-10, by macrophages and DCs. They also promote apoptosis of macrophages, DCs and T cells, leading to inhibition of immune responses (Sternberg, 2006).

1.2.4 GCs and Ovarian Cancer

Several studies have described high levels of cortisol in saliva, blood and ascites from ovarian cancer patients. In these patients, a dysregulation of diurnal cortisol levels leading to greater functional disability, fatigue and depression was assumed and implications for disease progression could not be excluded (Touitou et al., 1996; Lutgendorf et al., 2008; Weinrib et al., 2010).

Synthetic GCs such as dexamethasone are administered to patients with ovarian cancer as co-treatment with chemotherapy to prevent chemotherapy-related toxic side effects. GCs have potent anti-emetic effects, protect normal tissue and can prevent hypersensitivity reactions associated with paclitaxel therapy (Runnebaum and Brüning, 2005; Zhang et al., 2006; Sui et al., 2006; Melhem et al., 2009; Chen et al., 2010).

There is some evidence that stress can affect cancer progression (Sood et al., 2006; Thaker et al., 2006). Furthermore, stress hormones have been reported to increase the tissue expression of matrix metalloproteinases (MMP), key proteins involved in tumour cell penetration of the extracellular matrix and in tumour progression (Sood et al., 2006). Via these mediators, stress hormones are thought to promote the invasive and metastatic potential of ovarian carcinoma cells. Moreover, tumours in stressed animals showed significantly increased vascularization and angiogenesis due to up-regulation of expression of VEGF, MMP2 and MMP9, which resulted in enhanced tumour growth (Thaker et al., 2006).

As already mentioned, GCs are frequently used as a co-treatment in cytotoxic cancer chemotherapy with drugs like cisplatin and paclitaxel, in order to prevent the related toxic side effects e.g. to stop nausea and to guard normal tissue. However, several studies have reported that this use of GCs could render cancer cells more resistant to apoptosis induced via cytotoxic chemotherapy since GCs may also up-regulate expression of survival genes like the caspase inhibitor cIAP2. Thus, GCs may reduce the effectiveness of anti-tumour chemotherapy by promoting resistance and anti-apoptotic properties in epithelial ovarian cancer cells (Runnebaum and Brüning, 2005; Sui et al., 2006; Zhang et al., 2006; Chen et al., 2010).

1.3 Transforming growth factor-beta in Ovarian Cancer

Transforming growth factor-beta proteins (TGF-β) are a family of polypeptide cytokines which regulate cell growth, tissue remodeling, development, tissue recycling, repair and immune suppression (Massagué, 1996; Bartlett et al., 1997). The immune suppressive and tissue remodeling activities of these growth factors propose a role in tumour metastasis, while angiogenic effects may contribute to tumour growth (Bartlett et al., 1997).

Three forms of TGF-β have been identified, namely TGF-β1, TGF-β2 and TGF-β3, in homo-dimeric chains of between 111 and 113 amino acids, with molecular weights of 25 kDa. These growth factors act together with cell surface serine-threonine kinase linked receptors which mediate their tissue regulatory effects (Massagué et al., 1992; Wrana et al., 1994; Bartlett et al., 1997).

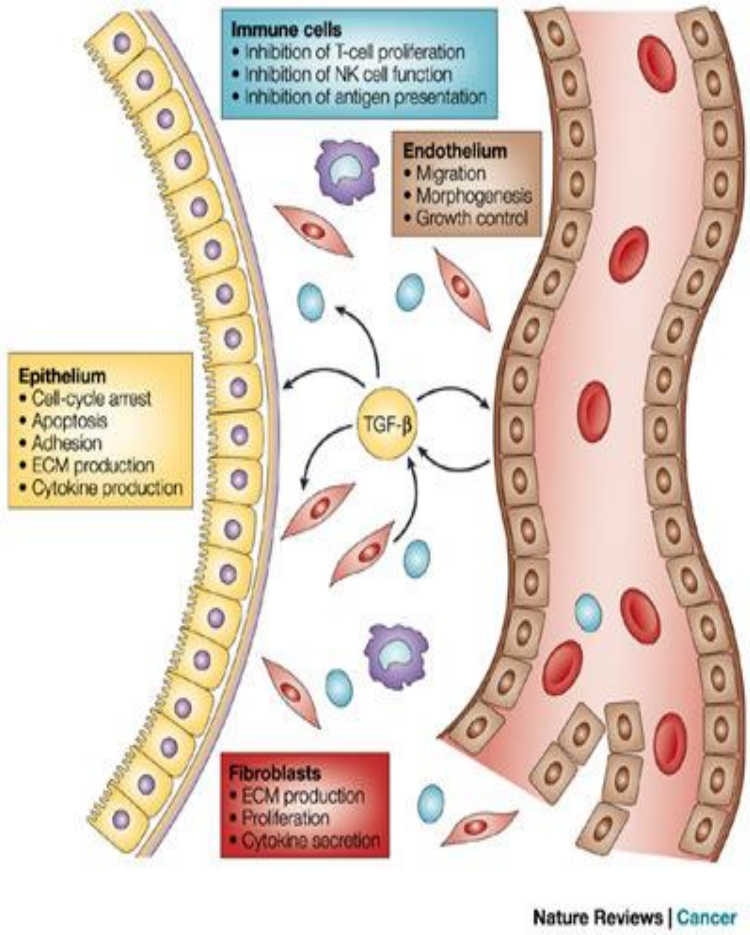


Figure (9): Targets of TGF-β and its actions in mature tissues. TGF-β helps to maintain tissue homeostasis by controlling the proliferation of various cell types including epithelial, endothelial, stromal fibroblasts and immune cells as well as by influencing the interaction of these cells with the tissue microenvironment (Siegel and Massagué, 2003).

TGF-β is released from every leukocyte lineage, including lymphocytes, macrophages, and dendritic cells (DCs) (Letterio and Roberts, 1998). Also, it has been mentioned that TGF-β is secreted by malignant cells, mesenchymal cells, regulatory T cells (T_{reg}), macrophages, neutrophils, NK T cells, monocytes, DCs, mast cells and platelets (Teicher, 2007).

TGF-β is a key player in malignant disease through its actions on host tissues and cells. Malignant cells mostly secrete large amounts of TGF-β that act on non-transformed cells present in the tumour mass as well as on distal cells in the host to suppress anti-tumour immune responses inducing an environment of immune tolerance, augmenting angiogenesis, invasion and metastasis, and increasing tumour extracellular matrix deposition (Teicher, 2007).

Local and systemic secretion of TGF-β as an immunosuppressive cytokines may have an important role in the impaired anti-tumour immune response commonly observed in advanced ovarian cancer. However, the observation that plasma levels of TGF-β are significantly higher than those detected in ascitic fluid, increases the possibility that cells other than tumour cells are in charge of release TGF-β release into the bloodstream of these patients (Santin et al., 2001).

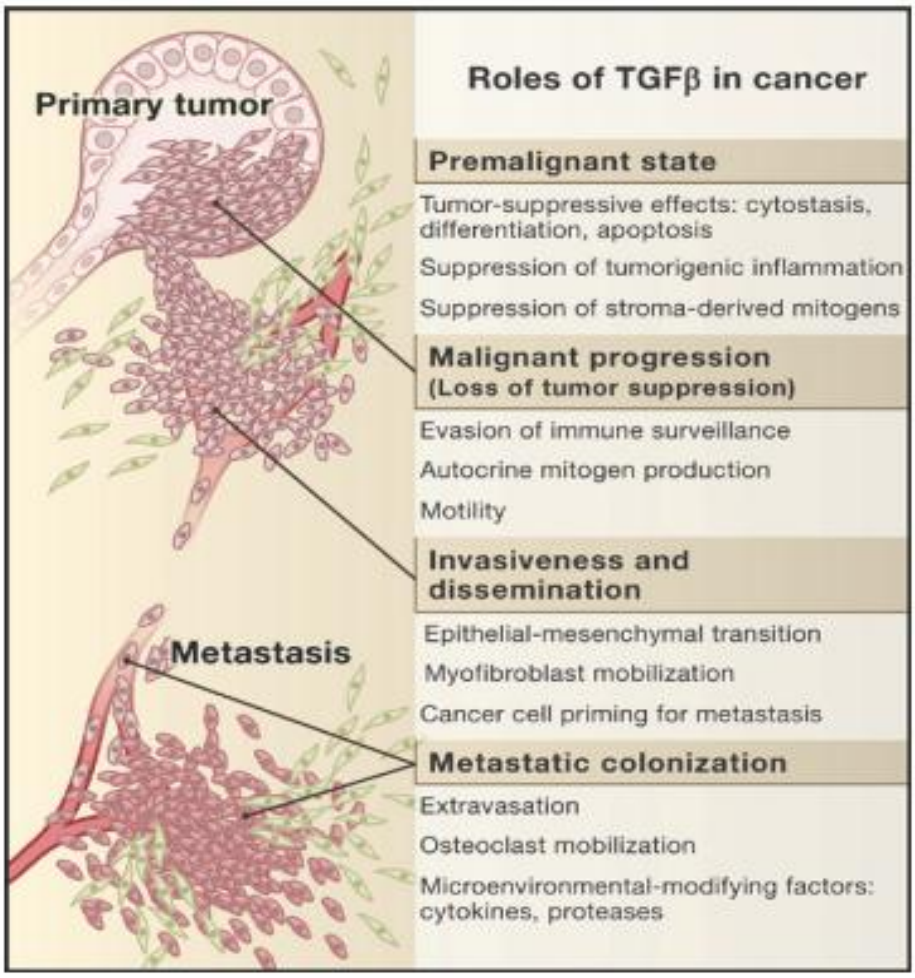


Figure (10): Role of TGF-β in Cancer. In normal and premalignant cells, TGF-β enforces homeostasis and suppresses tumour progression directly through cell-autonomous tumour-suppressive effects (cytostasis, differentiation, apoptosis) or indirectly through effects on the stroma (suppression of inflammation and stroma-derived mitogens). However, when cancer cells lose TGF-β tumour-suppressive responses, they can use TGF-β to their advantage to initiate immune evasion, growth factor production, differentiation into an invasive phenotype, and metastatic dissemination or to establish and expand metastatic colonies (Massagué, 2008).

Expression of TGF-β isoforms has been described in ovarian tumours (Henriksen et al.1995; Nash et al., 1999; Abendstein et al., 2000; Teicher, 2007) and high levels of TGF-β were also detected in ovarian cancer-associated ascites (Hirte & Clark 1991; Zeimet et al., 1998; Nash et al., 1999; Abendstein et al., 2000; Santin et al., 2001). In primary ovarian cancer and ascites, however, the respective distribution of the individual TGF-β isoforms was rather variable ranging from 46% (for TGF-β1) to 66% (for TGF-β2 and TGF-β3) of 74 malignant tumours (Bartlett et al., 1997). Still, irrespective of the isoform there is ample evidence that high levels of released TGF-β lead to disease progression (Merogi et al., 1996; Nakanishi et al., 1997; Abendstein et al., 2000).

1.4 GCs and TGF-β

GCs and TGF-β mutually contribute to limiting and/or terminating inflammatory processes and are both involved in the regulation of cell growth, development and differentiation. Moreover, GCs and TGF-β do not act separately of each other but modulate each other's activities (Periyasamy and Sánchez, 2002; Peltier et al., 2003). GCs up-regulate the expression of TGF-β receptor type II and improve the responsiveness and sensitivity of cells to TGF-β1 signaling (Li et al., 2006; Chen et al., 2010). Furthermore, GCs up-regulate TGF-β mRNA expression in human T lymphocytes (AyanlarBatuman et al., 1991; Peltier et al., 2003) and macrophages (Almawi and Irani-Hakime, 1998; Peltier et al., 2003). Moreover, dexamethasone also promotes the activation of latent TGF-β by inducing the secretion of lysosomal proteases, responsible for the cleavage of the latency-associated peptide (Oursler et al., 1993; Peltier et al., 2003). On the other hand, TGF-β1 alone not only increased cell adhesion and cell survival of HO-8910 cells in the presence of cisplatin, but also showed synergistic pro-adhesion and pro-survival effects with dexamethasone. Based on this observation, it was hypothesized that the ability of TGF-β signaling to increase adhesion of

human ovarian cancer cells to their extracellular matrix could significantly contribute to the previously described induction of chemo-resistance by GCs (Chen et al., 2010). It was further described that a TGF-β1-induced, Smad 2, 3- and AP-1-mediated process enhances glucocorticoid binding in inflammatory cells and thus improves responsiveness and sensitivity of these cells to glucocorticoids. Accordingly, the TGF-β pathway may represent a new target for intervention to increase glucocorticoid responsiveness (Peltier et al., 2003). Likewise, TGF-β potentiates the inhibitory role of dexamethasone on the growth of monocytic leukemia cells (Kanatani et al., 1996; Peltier et al., 2003).

1.5 Natural killer cells

Natural killer cells (NK cells) are large granular lymphocytes first described in 1975 as cells able to kill tumour cells *in vitro*. NK cells which constitute about 5-15% of the human peripheral blood lymphocytes from a healthy donor, are a vital part of the innate immune system and play a major role in the rapid immune responses against tumour cells and virus infected cells (Kiessling et al., 1975; Herberman et al., 1975; Riccardi et al., 1980; Ljunggren and Karre, 1985; Natuk and Welsh 1987; Salazar-Mather et al., 1998; Biron et al., 1999; Janeway, 5th Edition, 2005; Lodoen and Lanier; 2006).

Natural killer cells distinguish and lyse tumour cells and virus infected cells without previous priming to antigens (Herberman et al., 1975; Moretta et al, 2000; Terunuma et al., 2008), even though recent reports suggest that antigen-experienced NK cells can show superior efficacy (Sun et al., 2009; Paust and von Andrian, 2011). *In vivo* experiments have shown that NK cells can mediate resistance to tumour growth in mice and rejection of bone marrow transplants (Kiessling et al., 1975, 1977; Riccardi et al., 1980; Herberman and Ortaldo, 1981; Hanna, 1982). Moreover, NK cells play an important role against tumour promotion, growth and metastasis in rodents and humans (Smyth et al., 2002). Detection of human NK cells is usually based on identification of the lymphocytes that are negative for $CD3^-$ and positive for $CD16^+$ and $CD56^+$ (Terunuma et al., 2008). The most specific NK cell receptor, however, appears to be NKp46 (Walzer et al., 2007).

Upon stimulation of NK cells they release a number of cytokines, such as IFN-γ, TNF-α, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-5, IL-10 and IL-13 (Biron et al., 1999; Cooper et al., 2001; Moretta et al, 2001; Loza et al., 2002; Smyth et al., 2002;

Martin-Fontecha et al., 2004; Caligiuri, 2008; Terunuma et al., 2008). Moreover, cytokines and chemokines in the inflamed environment support the differentiation, proliferation and recruitment of NK cells (Smyth et al., 2002).

NK cells can establish contact with a target cells via CD16 which is a low-affinity activating Fc receptor that allows NK cells to bind to IgG-coated target cells, thus, inducing antibody-dependent cellular cytotoxicity (ADCC) as well as secretion of IFN-γ, TNF-α, and T- cells chemotactic recruiting cytokines (Trinchieri and Valiante, 1993; Lanier, 2005; Roda et al., 2006; Terunuma et al., 2008). However, NK cells can not only recognize "antibody-tagged" cells but also cells that express ligands for natural cytotoxicity receptors (explained in more detail below).

In spite of being part of innate immunity, NK cells are more closely related to T cells than to other leukocytes of the immune system. In fact, NK cells possess similar cytotoxic effector functions of cytotoxic T lymphocytes (CTL) and their cytokine-secreting properties resemble TH1 helper T cells (Lanier, 2005), Still, NK cells differ substantially from T cells which only recognize their cognate peptide antigen when presented on the right major histocompatibility complex (MHC). While this enables the exquisite antigen specificity of T cells, it comes at the price that no effective number of defenders against all possible antigens can be maintained in "standby mode". Thus, T cells require previous priming to antigens, gene rearrangement during development and clonal expansion before they can mount an effective immune response. NK cells, in contrast, constitute a first line of defense. Moreover, a successful NK cells response is accompanied by release of IFN-γ which then leads to activation of the adaptive immunity and initiates a Th1 immune response (Cooper et al., 2001; Martin-Fontecha et al., 2004; Raulet, 2004). Thereby, NK cells play an important role as link between the innate immunity and adaptive immunity to get rid of virus-infected or tumour cells by acting as mediator and effector cells in the immune system (Smyth et al., 2002). Furthermore, NK cells are considered to play a role in development of adaptive immunity through cross-talk with antigen-presenting DCs (Smyth et al., 2002).

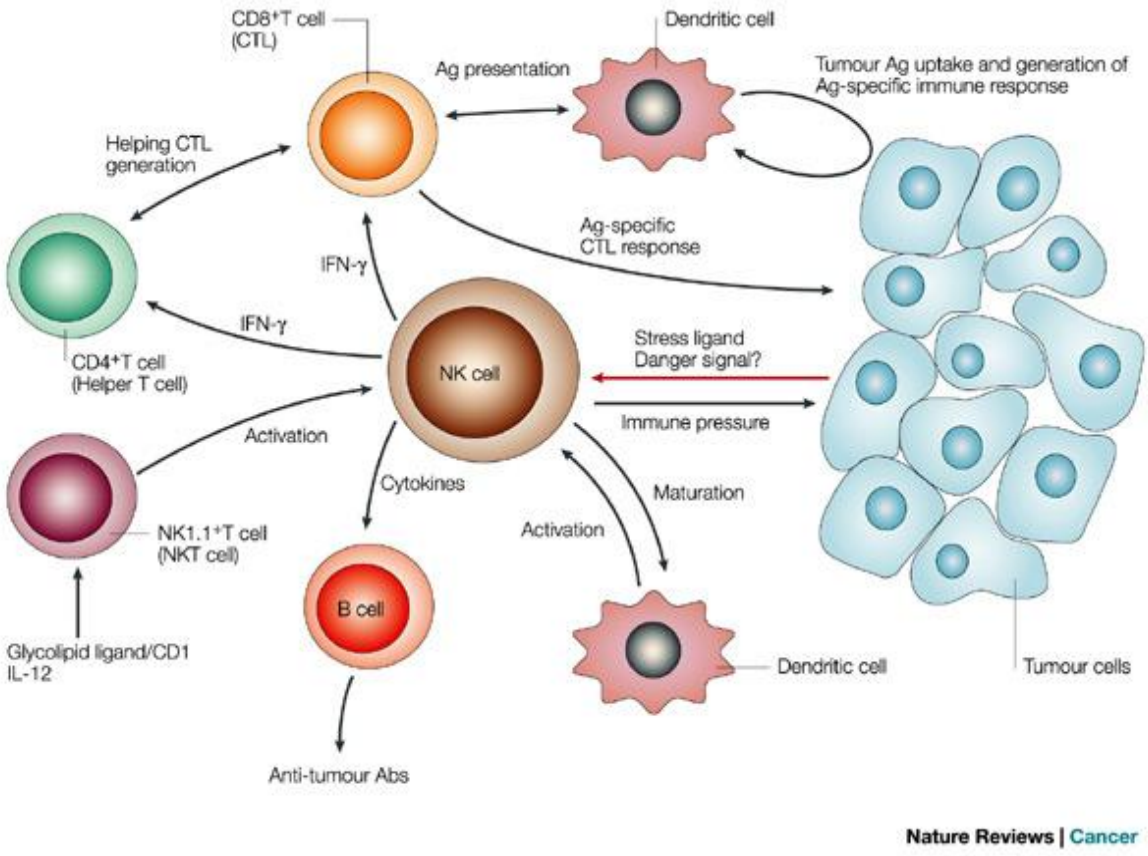


Figure (11): NK cells and anti-tumour immune responses (Smyth et al., 2002).

Natural killer cells are thought to play a major role in anti-tumour immunity and in networks of immune cells that respond to tumours (Fernandez et al., 1999; Smyth et al., 2002; Zamai et al., 2007). NK cells can not only lyse tumour cells directly (in response to stress-associate or antibody-mediated or other signals). They also influence the development of adaptive T- and B-cell immune responses which are instrumental for specific immunity and immunological memory to tumours and pathogens. NK cell-derived cytokines, such as interferon IFN-γ, activate CTL and helper T-cell ($CD4^+$) responses, leading to T cell proliferation and further cytokine production (Smyth et al., 2002).

NK cells can recognize tumour cells via different ligand-receptor interactions which result in the release of cytokines and in the formation of an immunological synapse with a target cell (Lanier, 2005). NK cells are further capable of lysing tumour cells via different effector mechanisms, including the expression of death ligands to activate the death-receptor pathway, the IFN-γ-mediated pathway and the exocytosis of cytolytic granules containing perforin and

granzymes (Smyth et al., 2002). The inner surface of these cytotoxic granules is coated with the lysosomal–associated membrane protein-1 (LAMP-1 or CD107a) which translocates to the surface of NK cells upon degranulation. The presence of LAMP-1 at the cell surface is thus considered as marker for activated NK cells (Peters et al., 1991; Fukuda, 1991; Alter et al., 2004).

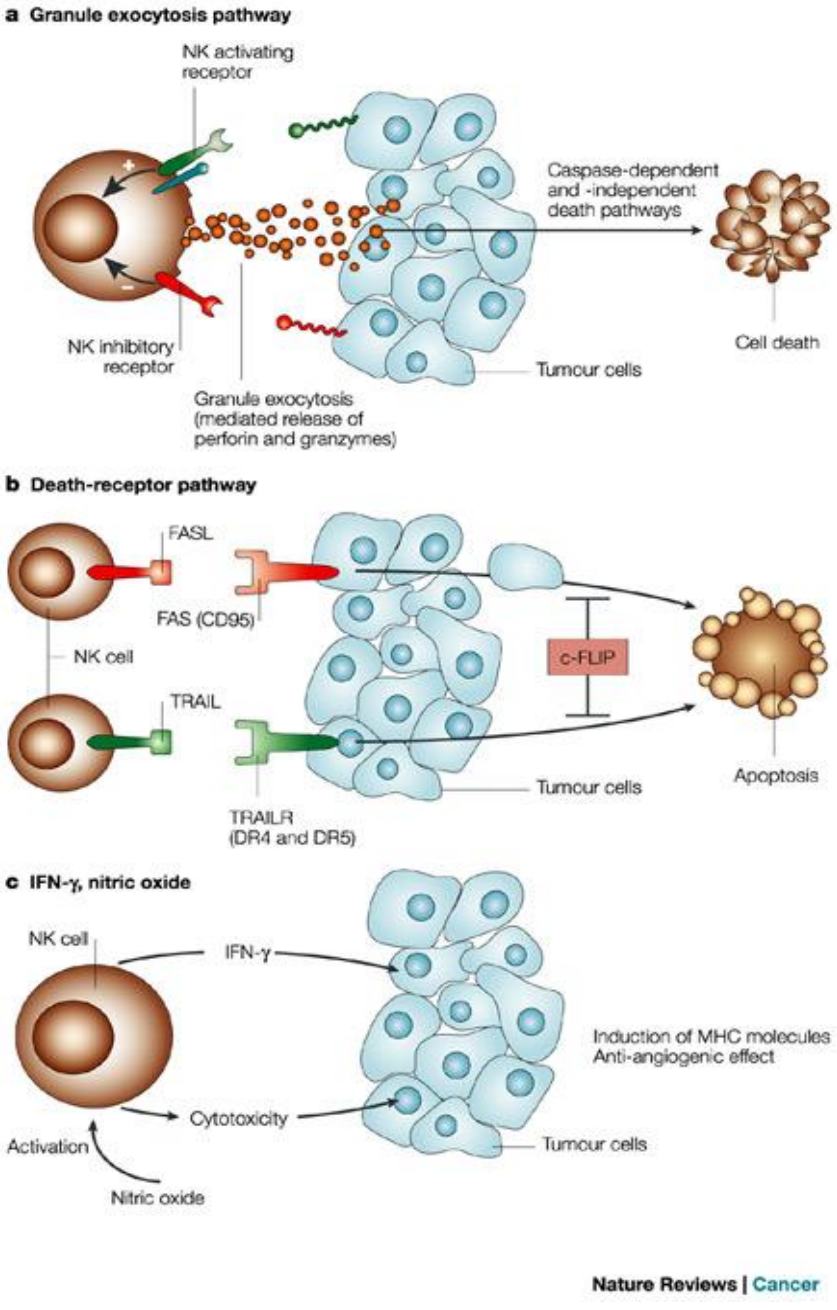


Figure (12): Role of NK cells in the elimination of tumour cells. NK cells can use the perforin/ granzyme granules exocytosis pathway (a), the death-receptor-ligand pathway (b) or the IFN-γ, nitric oxide pathway (c) to kill tumour cells (Smyth et al., 2002).

NK cells express a number of inhibitory receptors that recognize major histocompatibility complex (MHC) class I molecules which are expressed on normal cells. NK cells are able to distinguish between normal major histocompatibility complex class I positive (MHC class I^+) cells and cells that either lack or down-regulate their expression of MHC class I molecules as an outcome of tumour transformation or viral infection. The concept that NK cells lyse target cells which lack expression of one or more MHC class I alleles or that express insufficient amounts of class I molecules was called the "missing self hypothesis" by Ljunggren and Karre. Current models propose that NK cell functions are controlled by the balance of inhibitory and activating signals which they receive through their various classes of receptors (Ljunggren and Karre, 1985; Karre et al., 1986; Ljunggren and Karre, 1990; Moretta et al., 1996; Moretta et al., 2000; Moretta et al., 2001; Smyth et al., 2002). The link between NK cell triggering and inhibition is further emphasized by the fact that an efficient negative regulation of NK cell function requires the co-engagement of both activating and inhibitory receptors (Bléry et al., 1997; Moretta et al., 2000).

Interactions between activating receptors and their ligands on target cells enable NK to lyse a given target cell. Many of these activating ligands can be up-regulated upon infection or transformation of the cells or by other stress stimuli acting on cells (Groh et al., 1996; Bauer et al., 1999, Groh et al., 2001; Cerwenka et al., 2001; Moretta etal., 2000; Moretta etal., 2001; Lanier, 2005).

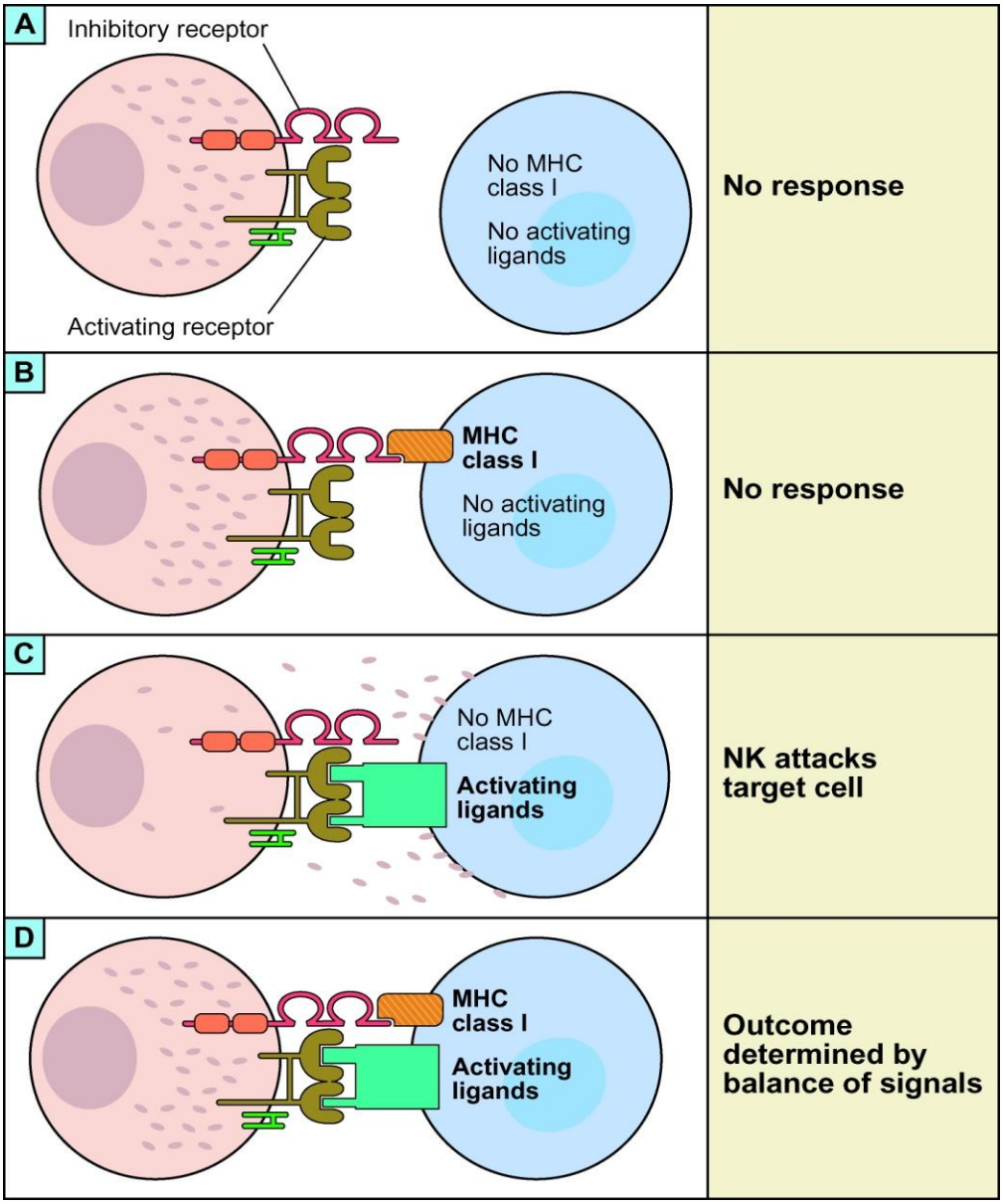


Figure (13): Missing-self hypothesis. The balance of inhibitory and stimulatory signals received by a natural killer cell determines the outcome of its interaction with a target cell. In case of NK cells interacting with target cells expressing ligands for both inhibitory and activating receptors, the outcome is determined by the summation of the strength of signals (Lanier, 2005).

1.5.1 Natural killer cell receptors

1.5.1.1 Inhibitory receptors

In human NK cells, both MHC class I molecule-specific (Moretta et al., 1996; Lanier, 1998; Long, 1999; Moretta et al., 2000) and non-classical MHC class I molecule-specific inhibitory receptors have been identified (Poggi et al., 1995; Meyaard et al., 1997; Cantoni et al., 1999; Falco et al., 1999; Moretta et al., 2000). Furthermore, the inhibitory receptors specific for HLA-class I belong to two structurally distinct families of molecules: the killer cell Ig-like receptors (KIR) and the killer cell lectin-like receptors (KLR). These inhibitory receptors prevent killing of normal cells and limit the production of inflammatory cytokines such as TNF-α, GM-CSF and IFN-γ. Since surface expression of HLA-class I molecules is changed in response to malignant transformation (Garrido et al., 1997; Moretta et al., 2000) or viral infection of the cells (Biron, 1997; Zeidler et al., 1997, Ploegh, 1998; Cohen., 1999; Moretta et al., 2000), the most important role of HLA-class I specific inhibitory receptors is to check the integrity of potential target cells and thereby prevent damage of normal tissues.

The inhibitory receptors include members of the KIR (Humans) and Ly49 (Mice) family which interact with MHC class I molecules as well as the killer cell lectin-like receptor (KLR) CD94/NKG2A which binds to non-conventional MHC I^b molecules (HLA-E in humans and Qa- I^b in mice) (Yokoyama, 1993; Lanier; 1998; Lanier, 2005; Raulet and Vance, 2006).

These inhibitory receptors signal via immunoreceptor tyrosine-based inhibitory motifs (ITIM). Upon receptor ligation, tyrosine residues in the ITIM domain (I/V/L/SxYxxL/V) are phosphorylated which enables them to interact with cytosolic Src-homology (SH2) domain-containing proteins such as SHP-1 which are thus recruited to the inhibitory receptor complex. This leads to considerable changes in gene expression and stops activating signals that might otherwise lead to cytokine release and cytotoxic effector activity (Lanier, 1998; Tomasello et al., 2000; Moretta et al., 2000; Lanier, 2005).

1.5.1.2 Activating NK receptors

In humans, several activating receptors expressed by NK cells (including NKp30, NKp44, NKp46, NKp80, NKG2D, DNAM-1, CD2, CD16 and 2B4) are responsible for cytotoxic activity of NK cells against transformed and virally infected cells (Moretta et al, 2000; Moretta et al, 2001; Lanier, 2005).

The first triggering NK receptors were found based on the screening of mAbs able to induce NK-mediated killing of Fc-γ receptor positive tumour target cells in a redirected killing assay (Moretta et al, 2001).

Natural cytoxicity receptors (NCRs) are selectively expressed by natural killer cells. NKp46 and NKp30 are expressed by resting and activated NK cells whereas NKp44 is expressed by activated NK cells only (Vitale et al., 1998; Moretta et al, 2000; Moretta et al, 2001). NKp80 is expressed by NK cells that are freshly isolated from peripheral blood as well as by a small T cell subset characterized by the $CD3^{+}D56^{+}$ surface phenotype (Moretta et al, 2001). Upon crosslinking, NCRs mediate NK-cell activation, leading to the killing of target cells and to cytokine production (Moretta et al, 2000; Moretta et al, 2001). The ligands for NKp46 and NKp44 are yet unidentified (Moretta et al, 2000; Moretta et al, 2001; Lanier, 2005), whereas the ligand for NKp80, activation-induced C-type lectin (AICL), was described a few years ago (Welte et al., 2006; Thomas et al., 2008). Likewise, nuclear factor HLA-B associated transcript 3 (BAT3) was identified as an activating ligand for NKp30 (Pogge von Strandmann et al., 2007; Simhadri et al., 2008). Still, it appears possible that further ligands may be discovered.

NKG2D is one of the activating receptors expressed on NK cells, γδ T cells, $CD8^{+}$ T cells and a small subset of $CD4^{+}$ T cells. It is encoded by the killer cell lectin-like receptor subfamily K, member 1 gene (KLRK1) (Bauer et al., 1999; Moretta et al, 2000; Moretta et al, 2001; Lanier, 2005). The NKG2D receptor recognizes cell surface glycoproteins that are structurally related to MHC class I. These ligands, however, are not encoded by genes in the MHC complex and are stress-inducible molecules expressed on the tumour cells and virally infected cells. Human NKG2D ligands include MICA, MICB and ULBP1-6; while mouse NKG2D ligands include RAE-1, H60 and MULT1 molecules (Moretta et al, 2000; Moretta et al, 2001; Lanier, 2005).

DNAM-1 (CD226) is one of the triggering receptors that are not restricted to NK cells only, but also expressed on T cells, B cells, monocytes, and platelets (Burns et al., 1985; Scott et al., 1989; Shibuya et al., 1996; Moretta et al, 2001; Lanier, 2005). The ligands of DNAM-1 receptor are CD112 (Polio virus receptor, PVR) and CD155 (Nectin-2) (Bottino et al., 2003; Tahara-Hanaoka et al., 2004; Lanier, 2005). Ligation of DNAM-1 by its ligand expressed on tumour cells provides a potent trigger for NK cell–mediated cytotoxicity and cytokine production (Masson et al., 2001; Bottino et al., 2003; Tahara-Hanaoka et al., 2004; Lanier, 2005).

2B4 (CD244) is another activating NK cell receptor and a member of the CD2 subfamily the members of which mainly functions as co-receptors rather than as true receptors. However, they can greatly enhance cytotoxic effector functions when co-engaged with a triggering receptor. 2B4 is expressed on human and mouse NK cells, γδ T cells, and $CD8^+$ T cells (Moretta et al, 2000; Moretta et al, 2001; Smyth et al., 2002; Lanier, 2005; Sinha et al., 2010). The triggering function of 2B4 on NK cells becomes evident upon ligation with its ligand CD48 when it is simultaneously co-engaged with triggering receptors (Moretta et al, 2000; Moretta et al, 2001; Smyth et al., 2002; Lanier, 2005; Raulet and Vance, 2006). Nevertheless, 2B4 may play a role as both inhibitory or stimulatory receptor, depending on the further receptors involved and on the associated signaling molecules in the respective cell type (Raulet and Vance, 2006). Since CD48 is expressed on the majority cells of hematopoietic cells, 2B4 on NK cells has the potential to interact with different cell types, including other NK cells. Thus, studies exploring the function of 2B4 in IL-2–propagated NK cells gave rather ambiguous results (Sinha et al., 2010).

2 Material and Methods

2.1 Material

2.1.1 Devices

Device	Manufacturer or distributor
ABI TaqMan 7500	Applied Biosystems, Life Technologies Corporation, Carlsbad, California 92008, USA
Agarose gel electrophoresis apparatus	MupiD-exU, Eurogentec GmbH, Cologne, Germany
Autoclave	H P Labortechnik AG, 85764 Oberschleißheim, Germany
Balance	Sartorius AG, 37075 Goettingen, Germany
Centrifuges	Eppendorf, 22339 Hamburg, Germany
	Jouan/Thermo Electron GmbH, 63303 Dreieich, Germany
CO_2 Incubator	Thermo Electron GmbH, 63303 Dreieich, Germany
Counting chamber	Carl Roth GmbH + Co. KG 76185 Karlsruhe, Germany
Cryo box	Nalgene, Thermo Fisher Scientific, 63505 Langenselbold, Germany
Digital camera	Canon, USA
ELISA-Reader Sunrise	TECAN, 74564 Crailsheim, Germany
FACSCalibur	Becton Dickinson, Franklin Lakes, NJ 07417, USA
Freezers (-20°C, -86°C)	Liebherr, Germany ; Thermo Electron GmbH

	63303 Dreieich, Germany
	Philipp Kirsch GmbH. 77608 Offenburg, Germany
Heating block	Biometra GmbH, D-37079 Goettingen, Germany
Hot water bath	Julabo, 77960 Seelbach, Germany
Ice machine	Scotsman Ice Systems, Vernon Hills, IL 60061, USA
Inverted Microscope	Leica, 35606 Solms, Germany
Irradiation machine	Philips Electronics, Germany
Laminar flow hood	Heraeus, Hanau, Germany
Liquid nitrogen tank	Air Liquide, 40235 Düsseldorf, Germany
Luminometer Orion II	Berthold Detection Systems, Germany
MACS™ Separators	Miltenyi Biotec GmbH, 51429 Bergisch Gladbach, Germany
Magnetic stirrer	VWR, 64295 Darmstadt, Germany
Microwave	Sharp Electronics (Europe) GmbH, Germany
Olympus IX-70 Inverted System Microscope	Olympus, 20097, Hamburg
PCR thermocycler	Biometra GmbH, D-37079 Goettingen, Germany
pH-Meter	Mettler-Toledo GmbH, 35353 Gießen, Germany
Photometer	Thermo Electron GmbH, 63303 Dreieich, Germany

Pipettes	Eppendorf AG, 22339 Hamburg, Germany
Power-Supply	Thermo Electron GmbH, 63303 Dreieich, Germany
Refrigerator	Liebherr, Germany
Roller mixer	Stuart, Bibby Scientific Limited, Beacon Road, Stone, Staffordshire ST15 0SA, UK
Sample shaker (Vortex)	VWR, 64295 Darmstadt, Germany
SDS gel electrophoresis system	Whatman, GE Healthcare, D-80807 Munich, Germany
Shakers	Heidolph Instruments GmbH & Co. KG, 91126 Schwabach, Germany
Thermo-mixer	Eppendorf AG, 22339 Hamburg, Germany
Ultra Freezer (-86°C)	Heraeus, Hanau, Germany
UV lamp	Biometra GmbH, D-37079 Goettingen, Germany
X-ray film Cassettes	Carl Roth GmbH + Co. KG, 76185 Karlsruhe, Germany

2.1.2 Consumables

Consumables	**Manufacturer or distributor**
X-ray films	Fisher Scientific GmbH - Im Heiligen Feld 17 - D-58239 Schwerte, Germany
21-gauge needle	Becton Dickinson, Franklin Lakes, NJ 07417, USA
Cell culture dishes	TPP, 8219 Trasadingen, Switzerland

Cell culture flasks (25 cm^2, 75 cm^2)	Greiner bio-one, 72636 Frickenhausen, Germany
Cell culture plates (96-well, 24-well, 12-well, 6-well	TPP, 8219 Trasadingen, Switzerland
Cryotubes	Nunc, Roskilde, Denmark
FACS tubes	Hartenstein, 97078 Würzburg, Deutschland
Falcon tubes (15 ml, 50 ml)	Greiner bio-one, 72636 Frickenhausen, Germany
Film (TaqMan)	Sarstedt, 51588 Nürmbrecht, Deutschland
Gloves	Cardinal Health, 7000 Cardinal Place, Dublin, OH 43017, Ireland
Injection cannula	B. Braun Melsungen, Pfieffewiesen 34212 Melsungen, Germany
KODAK developer	KODAK
KODAK fixer	KODAK
LS columns	Miltenyi Biotec GmbH, 51429 Bergisch Gladbach, Germany
Luminometer plates	Greiner bio-one, 72636 Frickenhausen, Germany
Micro test tubes (Eppendorf caps)	Greiner bio-one, 72636 Frickenhausen, Germany
Microtiter plates (Maxisorp)	Nunc, Roskilde, Denmark
Nylon Cell Strainer 70µm	Becton Dickinson, Franklin Lakes, NJ 07417, USA
Parafilm	Pechiney, 8770 W. Bryn Mawr Ave. Chicago, IL
PCR tubes (8-Stripes, 96-well	Sarstedt, 51588 Nürmbrecht, Deutschland
plate ABITaqMan-Platte)	

Perfusor syringe	B. Braun Melsungen, Pfieffewiesen 34212 Melsungen, Germany
Petri dishes	Carl Roth GmbH + Co. KG, 76185 Karlsruhe, Germany
Pipette tips (10 µl, 100µl, 100µl)	Sarstedt, 51588 Nürmbrecht, Deutschland
PVDF membrane	Carl Roth GmbH + Co. KG, 76185 Karlsruhe, Germany

2.1.3 Cell lines

Cell line	Species	Origin
OaW-42	Human, OvCa	European Collection of Cell Cultures (ECACC)
OVCAR-3	Human, OvCa	ATCC Manassas, VA 20110-2209, USA
PA1	Human, OvCa	ATCC Manassas, VA 20110-2209, USA
RPMI 8866	Human, lymphoma	ATCC Manassas, VA 20110-2209, USA
SKOV-3	Human, OvCa	ATCC Manassas, VA 20110-2209, USA
K562 (pCDNA3.1-fluc)	Human erythroleukemic	Dr. Ruth Seggewiß, Department of Internal Medicine, University of Würzburg

2.1.4 Plasmid

Plasmid	Gene expression	Origin
CMV-fluc	Firefly-luciferase	Dr. Michael Jensen (City of Hope national medical center and Beckman Research Institute, Duarte, Ca, USA).

2.1.5 General reagents for cell culture

Media , solutions and media additives	Manufacturer or distributor
Dimethylsulfoxid (DMSO)	Carl Roth
Dulbecco's PBS	PAA
Fetal calf serum (FCS)	PAA
Penicillin/Streptomycin	PAA
RPMI 1640	PAA

Buffers and solutions	Composition
50 x TAE	2 M Tris, 0.6 M EDTA, 0.57% glacial acetic acid
6 x DNA- loading buffer	30% Glycerin, 50 mM EDTA, 0.001% bromophenol blue
FACS-Buffer	PBS, 2% FCS
Freezing medium	70% FCS, 20% standard medium, 10 % DMSO
MACS-Buffer	PBS pH 7.2, 0.5% BSA, 2 mM EDTA
PBA	PBS, 1% BSA
PBS	37 mM NaCl, 2.7 mM KCl, 80 mM Na_2HPO_4, 1.8 M KH_2PO_4, pH 7.4
PBS-T	PBS, 0.05% Tween20
Standard medium	88.5% RPMI 1640, 10% FCS, 1% P/S
TBS	10 mM Tris-HCl, 150 mM NaCl, pH 7.5

2.1.6 Buffers for polyacrylamide gels

Gel	Composition
Separating gel 10%	dd H_2O 1.9 ml, Acrylamid/Bisacrylamid (30%) 1.7 ml , Tris-HCl 1.5 M, pH 8.8 1.3 ml , SDS (10%) 50 µl, APS (10%) 50 µl, TEMED 2 µl
Stacking gel 5%	dd H_2O 0.68 ml, Acrylamid/Bisacrylamid (30%) 0.17 ml, Tris-HCl 1 M, pH 6.8 0.13 ml, SDS (10%) 10 µl, APS (10%) 10 µl, TEMED 1 µl

2.1.7 Buffers for SDS-PAGE and Western blot

Buffer	Composition
Blocking Buffer	5% skim milk powder in PBS-T
ECL solution A	50 mg Luminol in 200 ml 0.1 M Tris-HCl pH 6.8
ECL solution B	11 mg para- hydroxycoumarinic acid in 10 ml of DMSO
Lysis buffer	50 mM Tris-HCl, pH 8, 120 mM NaCl, 5mM EDTA, 0.5% NP-40, 2 µg/ml, Aprotinin, 10µg/ml Leupeptin, 100 µg/ml PMSF, 50 mM NaF, 200 µM $NaVO_5$
Neutralization Buffer	1.5 M Tris-Base pH 7.4
PBS-T	PBS, 0.05% Tween20
Ponceau S solution	0.2% Ponceau S in 3% trichloroacetic acid
Protein loading buffer (5x)	100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% Bromophenol blue, 20% Glycerol, 10% β-Mercaptoethanol

Running buffer	25 mM Tris. 193 mM Glycin, 0.5% SDS pH 8.8
Stripping Buffer	0.2 M Glycin, 0.5 M NaCl, pH 2.8
Transfer buffer	25 mM Tris, 192 mM Glycin, 20% Methanol

2.1.8 Reagents for FACS staining

Reagent	Manufacturer or distributor
Beriglobin solution	Novartis
FACS Clean	Becton Dickinson
FACS Flow	Becton Dickinson
FACS Rinse	Becton Dickinson
Monensin	BioLegend

2.1.9 Detachments

Reagent	Manufacturer or distributor
Accutase	PAA
Trypsin EDTA	PAA

2.1.10 Kits

Kit	Manufacturer or distributor
ABsolute Blue QPCR SYBR Green low Rox mix	Thermo Fisher Scientific
CD90.2 MicroBeads Kit	MiltenyiBiotec
Cell Proliferation Reagent WST	Roche
Cortisol luminescent immunoassay	IBL
Crimson longAmp Polymerase Kit	New England Biolabs
iScript cDNA Synthesis Kit	Biorad
MACS NK Cell Isolation Kit II	MiltenyiBiotec

2.1.11 Disinfectant

Disinfectant	Manufacturer or distributor
Cutasept	Bode
Ethanol (EtOH)	Hospital pharmacy

2.1.12 Cytokine

Cytokine	Concentration	Manufacturer or distributor
IL2	100 U/ml	PeproTech
TGF-β	5 ng/ml	PeproTech

2.1.12 Virus

Virus	Source
AdCre virus	University of Iowa GeneTransfer Vector Core

2.1.13 Oligonucleotides

Oligonucleotide	Origin	Sequence 5´ -> 3´
18s RNA, forward	Sigma	CGGCTACCACATCCAAGGAA
18s RNA, reverse	Sigma	GCTGGAATTACCGCGGCT
GAPDH, forward	Eurogentec	ACGACAGTCCATGCCATCAC
GAPDH, reverse	Eurogentec	TCCACCACCCTGTTCCTGTA
h11β-HSD1, forward	Sigma	ATTCACCATGTGCGCAAAAG
h11β-HSD1, reverse	Sigma	GGAGACGACAACAATGCTTCC
h11β-HSD2, forward	Sigma	GGGCCTATGGAACCTCCAA

h11β-HSD2, reverse	Sigma	GACCCACGTTTCTCACTGACTCT
h2B4, forward	Sigma	ACAAAAGGCCACATTCCAAG
h2B4, reverse	Sigma	AACCCAGAGAGGGGAGAAAA
hCD2, forward	Sigma	CCAGCCTGAGTGCAAAATTCA
hCD2, reverse	Sigma	GACAGGCTCGACACTGGATTC
hDNAM-1, forward	Sigma	GCAGTGACCAAGAGGGTGTT
hDNAM-1, reverse	Sigma	CTGCTCGCTCTAACGCTTCT
hNKG2D, forward	Sigma	TCTCGACACAGCTGGGAGATG
hNKG2D, reverse	Sigma	GACATCTTTGCTTTTGCCATCGTG
hNKp30, forward	Sigma	TGTCCTGAGAAATGGGAAGG
hNKp30, reverse	Sigma	ACAGTGTTCAGGGACCCAAG
hNKp46, forward	Sigma	ACAACCATGCCTGGTCTTTC
hNKp46, reverse	Sigma	AAAAGGTAGCGTGCCCCAAGT
m11β-HSD1, forward	Sigma	GTCCCTGTTTGATGGCAGTT
m11β-HSD1, forward	Sigma	TTATGAAAAAATACCTCCTCCC
m11β-HSD1, reverse	Sigma	GCCCCAGTGACAATCACTTT
m11β-HSD1, reverse	Sigma	CTTTGATCTCCAGGGCGCATTC
mGR, forward	Sigma	GTGAGTTCTCCTCCGTCCAG
mGR, reverse	Sigma	TACAGCTTCCACACGTCAGC
mIL-1α, forward	Sigma	CAAACTGATGAAGCTCGTCA
mIL-1α, reverse	Sigma	TCTCCTTGAGCGCTCACGAA
mIL-1β, forward	Sigma	CTGTGTCTTTCCCGTGGACC

mIL-1β, reverse	Sigma	CAGCTCATATGGGTCCGACA
mKras dt5 new	Sigma	GTCGACAAGCTCATGCGGG
mKras SD5 new (5 Mut)	Sigma	CCATGGCTTGAGTAAGTCTGC
mKras Uni new(3 Universal)	Sigma	CGCAGACTGTAGAGCAGCG
mMMP-9, forward	Sigma	CGGCACGCTGGAATGATC
mMMP-9, reverse	Sigma	TCGAACTTCGACACTGACAAG
mPten6637, forward	Sigma	TCCCAGAGTTCATACCAGGA
mPten6925,reverse	Sigma	GCAATGGCCAGTACTAGTGA
mPten7319,reverse	Sigma	AATCTGTGCATGAAGGAAC
mTNF-α, forward	Sigma	GGGCCACCACGCTCTTC
mTNF-α, reverse	Sigma	GGTCTGGGCCATAGAACTGAT

2.1.13 Chemicals, solutions and reagents

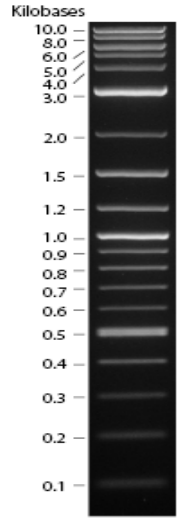
Chemicals, solutions and reagents	Manufacturer or distributor
ACD-A (sodium citrate solution)	Haemonetics
Acetone	Carl Roth
Acrylamid/ Bisacrylamid	Carl Roth
Agarose	Carl Roth
Ammonium persulfate (APS)	Carl Roth
Aprotinin	Carl Roth
Aqua ad iniectabilia	Braun
Biocoll separation solution	Biochrom
Bovines Serum Albumin (BSA)	Applichem
Bromophenol blue	Carl Roth
Chloroform	Sigma

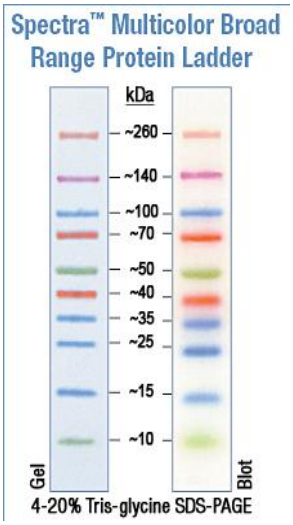
Cortisol	Rotexmedica
Diaminobenzidine (DAB)	Dako
Dispase	Roche
Ethylenediaminetetraacetic acid (EDTA)	Applichem
GelRed	Biotium
Glacial acetic acid	Carl Roth
Glycerin	Carl Roth
Glycin	Carl Roth
Goat serum	Caltag
Haematoxylin	Carl Roth
Human serum albumin (HSA)	Behring
Hydrochloric acid	Applichem
Hydrogen peroxide	Carl Roth
Ionomycin	Cayman
Isopropanol	Carl Roth
Leupeptin	Carl Roth
Luciferin	P.J.K.
Luminol salt	Sigma
Methanol	Hospital pharmacy
NP-40	Applichem
PeqGold Trifast	Peqlab
Phenylmethanesulfonylfluoride (PMSF)	Carl Roth
Phorbol 12-myristoyl 13-acetate (PMA)	Sigma
Phospho-Stop (tablets)	Roche
Ponceau S	Carl Roth
Potassium (di) hydrogen phosphate	Carl Roth
Potassium chloride	Carl Roth

Roti-Quant	Carl Roth
Skimmed milk powder	Merck
Sodium (di) hydrogen phosphate	Carl Roth
Sodium chloride	Carl Roth
Sodium dodecyl sulfate (SDS)	Carl Roth
Sodium fluoride	Applichem
Sodium hydroxide	Carl Roth
Sodium orthovanadate	Applichem
Streptavidin-HRP	Dako
Tetramethylethylenediamine (TEMED)	Carl Roth
Trichloroacetic acid	Carl Roth
Tris hydroxymethyl aminomethan (Tris)-Ultra	Carl Roth
Tris-HCl	Carl Roth
Tween20	Carl Roth
Vitro Clud	Langenbrinck
β-Mercapthoethanol	Carl Roth

2.1.14 Standard DNA and protein ladder

TriDye™ 2-Log DNA Ladder (0.1 - 10.0 kb) Thermo Fischer Scientific New England Biolabs





2.1.15 Antibodies

Antibody	Species	Label	Dilution	Source	Clone or Catalog No.
Anti- human 11β-HSD1	Rabbit	Purified	1:50	Abcam	ab39364-100
Anti- human 11β-HSD2	Rabbit	Purified	1:50	Biotrend	BHSD23-A
Anti- human CD14	Mouse	Purified	1:50	Dako	TÜK4
Anti- human CD68	Mouse	Purified	1:50	AbD Serotec	514H12
Anti- human CD85	Mouse	Purified	1:50	Dako	VMP55
Anti- human EpCAM	Mouse	Purified	1:50	MiltenyiBiotec	HEA-125
Anti- human GR	Rabbit	Purified	1:1000	Santa Cruz	Sc-8992 (H-300)
Anti- sheep IgG (Secondary antibodies)	Rabbit	HRP	1:5000	Santa Cruz	Sc- 2770
Anti-human 2B4	Mouse	Purified	1:500	BioLegend	C1.7
Anti-human CD107a	Mouse	PE	1:20	BioLegend	H4A3
Anti-human CD2	Mouse	PE	1:100	MiltenyiBiotec	LT2
Anti-human CD3	Mouse	PE	1:100	ImmunoTools	MEM-57
Anti-human CD3	Mouse	FITC	1:100	ImmunoTools	MEM-57
Anti-human CD56	Mouse	APC	1:250	Beckman Coulter	N901
Anti-human DNAM-1	Mouse	FITC	1:100	eBioscience	11A8
Anti-human IgG1-isotype	Mouse	FITC	1:100	BioLegend	MOPC-21
Anti-human IgG1-isotype	Mouse	PE	1:100	BioLegend	MOPC-21
Anti-human NKG2D	Mouse	Purified	1:500	BioLegend	1D11
Anti-human NKG2D	Mouse	PE	1:100	MiltenyiBiotec	BAT221
Anti-human NKp30	Mouse	PE	1:100	MiltenyiBiotec	AF-29-4D12

Anti-human NKp44	Mouse	PE	1:100	MiltenyiBiotec	2.29
Anti-human NKp46	Mouse	PE	1:100	MiltenyiBiotec	9E2
Anti-mouse 11β-HSD1	Rabbit	Purified	1:50	Cayman	10004303
Anti-mouse 11β-HSD2	Sheep	Purified	1:50	Chemicon	AB1296
Anti-mouse CD4	Rat	Purified	1:50	ImmunoTools	YTS 191.1.2
Anti-mouse CD45.1	Mouse	PE	1:100	BioLegend	A20
Anti-mouse CD45.2	Mouse	FITC	1:100	BioLegend	104
Anti-mouse DCIR2	Rat	Biotin	1:50	MiltenyiBiotec	33D1
Anti-mouse EpCAM	Rat	Biotin	1:1000	BioLegend	G8.8
Anti-mouse FOXP3	Mouse	APC	1:50	MiltenyiBiotec	3G3
Anti-mouse IgG (Secondary antibodies)	Donkey	Alexa 488	1:50	Invitrogen	A-21202
Anti-mouse –IgG (Secondary antibodies)	Horse	HRP	1:5000	Cell signaling	#7076
Anti-mouse Pten	Rabbit	Purified	1:1000	Epitomics	EP229
Anti-mouse β-actin	Mouse	Purified	1:1000	Abcam	Ab8226
Anti-Rabbit IgG (Secondary antibodies)	Goat	HRP	1:5000	Cell signaling	#7074
Anti-Rabbit IgG (Secondary antibodies)	Goat	Alexa 555	1:50	Invitrogen	A-21428
Anti-Rat IgG (Secondary antibodies)	Goat	HRP	1:5000	Cell signaling	#7077

2.2 Methods

2.2.1 Thawing of cells

Cryopreserved cells were thawed quickly, resuspended in 1 ml of pre-warmed medium, transferred into a 15 ml tube containing 5 ml of pre-warmed medium and spun down for 5 min at 1,500 rpm. The supernatant was aspirated using a Pasteur pipette. Then, the cell pellet was resuspended in 5 ml fresh medium and transferred into a 25 cm^2 cell culture flask. During growth periods, the cells were cultured at 37°C/5% CO_2 and 95% relative humidity in the incubator.

2.2.2 Culturing and Sub-culturing of tumour cells

After growth in 25 cm^2 tissue culture flasks, cells were transferred to 75 cm^2 tissue culture flasks and cultured in 10 ml of medium at 37°C/5% CO_2 and 95% relative humidity. Medium change and subculture were performed when cells became confluent every three to four days. For sub-culturing, the cells were washed once with PBS before being incubated with 1 ml Trypsin/EDTA or Accutase. After 5-10 min at 37°C, the cells were completely detached. Then, the action of trypsin/EDTA or Accutase was stopped with 5 ml of FCS-containing medium, the cells were collected and centrifuged at 1500 rpm for 5 min, resuspended in 10 ml of medium and cells were diluted 1 to 10 and transferred into a new cell culture flask for further cell culture.

2.2.2 Cryopreservation of cells

For long-term archiving, cells were stored in a liquid nitrogen tank. For cryopreservation, the cells were detached with Trypsin/EDTA or Accutase (as described above), centrifuged at 1,500 rpm for 5 min and the cell pellet was taken up in 1 ml of fresh freezing medium. The cells were then placed for 24 h in a -80°C freezer equipped with special cryogenic boxes in which the temperature decreases by about 1°C/min. This enables a slow and gentle cell freezing to minimize cell death. After 24 h, the cells were transferred from the Cryobox into the liquid nitrogen tank.

2.2.3 Preparation and isolation of NK cells (Effector cells)

2.2.3.1 Isolation of human peripheral lymphocytes from whole blood (McGuckin et al., 2008)

To obtain peripheral blood lymphocytes (PBL), blood was drawn from healthy donors. To prevent clotting, 10% of sodium citrate solution was added, e.g. 5 ml of sodium citrate solution for 45 ml of whole blood. All steps were performed under sterile conditions. The citrated blood was diluted with twice the amount of PBS and distributed into 50 ml centrifuge tubes which had already been filled with 15 ml Biocoll lymphocyte separation medium. Without mixing, the citrate blood-PBS mixture was gently poured on the Biocoll layer in order to obtain two phases. For the following density gradient centrifugation, the centrifuge had to be at room temperature without brake and with very low acceleration. First, the tubes were centrifuged at 600 rpm for 20 minutes before; the uppermost 5 ml were aspirated from each tube to remove the platelets. The following centrifugation run lasted 30 minutes at 1400 rpm. Then, peripheral blood monocytes (PBMC) were ready to be collected from the interphase layer.

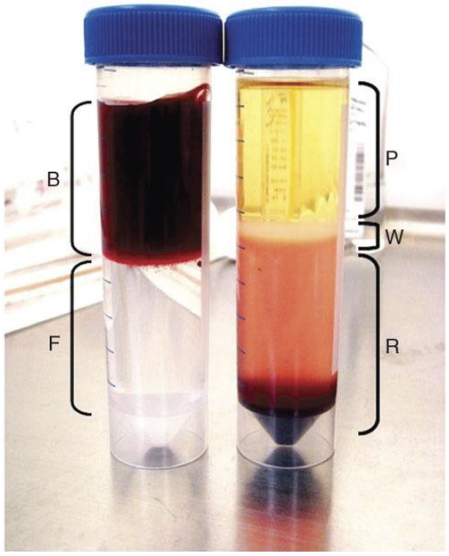


Figure (14): Scheme of the density gradient.

Left, before centrifugation: B = Blood-Citrate-PBS mixture
F = Biocoll Separation Solution
Right, after centrifugation, P = Plasma, and platelets
W= PBMC layer
R= Biocoll above erythrocytes

The PBMC layers of all tubes were then collected by gentle aspiration with a 5 ml pipette and transferred into fresh 50 ml tubes. Care was taken in order to minimize the amount of biocoll solution transferred with the cells. Then, the cells were washed and centrifuged twice with PBS for 8 minutes at 1,800 rpm. Afterwards, the PBMC cell pellet was resuspended and the cell number was counted with trypan blue in a Neubauer counting chamber. Approximately 50-60 million of these PBMCs were then resuspended in 10 ml of medium, placed in a cell culture dish and left to adhere for 1 h in an incubator. To obtain peripheral blood lymphocytes (PBL), the free floating cell fraction of was thereafter aspirated carefully while the cells that adhered to the floor of the cell culture dish represented mainly monocytes that could be used for other experiments. Typically, 20 -50 million PBL could be obtained from 50 ml of citrated blood.

2.2.3.2 Preparation of polyclonal NK cell cultures (Perussia et al., 1987)

Cultured RPMI 8866 feeder cells were irradiated with 30 Gy in a gamma irradiation facility (Neurological University Clinic Würzburg). Using 24-well plates, feeder cells and PBL were mixed in the ratio 1:4 in 500 µl RPMI medium per well and incubated for 8-14 days. On day 6 of culture, 500 µl of fresh medium was supplemented to each well. Cytokines and growth factors produced from feeder cells stimulate the clonal expansion of NK cells. This was observed by cell-cluster formation. The obtained polyclonal NK cell cultures were used for experiments between day 8 and 11. The purity of the polyclonal NK cell cultures was determined by flow cytometric staining for surface markers $CD3^{-}/CD56^{+}$.

2.2.3.3 Isolation of NK cells from whole blood by magnetically-activated cell sorting (MACS) technology

PBL were isolated, counted and resuspended very well to obtain a single cell suspension before they were centrifuged again at 300 ×g and 8°C for 10 min. The labeling and separation of the cells was carried out according to instructions provided with the "NK cell isolation kit II" manufactured by Miltenyi. With this kit, all except the desired cells were labeled with biotinylated antibodies against the corresponding surface markers. Subsequently, iron-containing anti-biotin beads were added to the biotinylated cell mixture. The labeled immune cells were loaded onto an LS column that was placed in a magnetic field in which the labeled immune cells were retained. The “untouched” fraction which passed through the column

consisted of the desired NK cells. After the column had been washed three times with MACS buffer, the purified cells were centrifuged at 1500 rpm for 5 min, counted and the purity was determined by flow cytometry staining for $CD3^-/CD56^+$ cells.

2.2.4 Flow cytometric analysis of immune cells

2.2.4.1 Extracellular FACS staining

Surface marker expression of immune cells was determined by flow cytometric staining. Cells were transferred into FACS tubes and washed once with PBS. The non-specific binding sites for antibodies (Fc-γ receptors) were blocked with human IgG (Beriglobin) solution. After washing once in FACS buffer, immune cells were incubated for 30 min in the dark with fluorescence-labeled antibodies against the respective surface antigens. Appropriate antibody concentrations had been determined via titration. After washing twice in FACS buffer, the stained cells were resuspended in 100-150 µl of FACS buffer. Flow cytometric analysis was performed on a FACS Calibur and evaluated using the Summit analysis program (Beckman Coulter, Version 4.3).

2.2.4.2 Detection of CD107a (LAMP-1) at the cell surface (Alter et al., 2004) to measure degranulation

Immune cells were isolated and treated for 72 h with 5 µM cortisol or 5 ng/ml TGF-β1 or both., As positive control, T cells were activated with 5 ng/ml PMA and 1µM Ionomycin. Then, CD107a antibodies (1:50 dilution) were added directly to the immune cells into the medium, followed by monensin (1:1000, 6µg/ml) to block the Golgi apparatus. Thereafter, cells were incubated for further 4 h at 37 °C, transferred to FACS tubes and washed twice with FACS buffer. For extracellular staining, the PBLs were blocked with Beriglobin solution at 4°C for 15 min before the surface markers CD3 and CD56 were counter-stained at 4°C for 30 min in the dark. After washing twice in FACS buffer, the stained cells were resuspended in 100-150 µl for flow cytometric analysis and analysed on a FACSCalibur.

2.2.5 Functional bioassays

2.2.5.1 Treatment of immune cells with cortisol and TGF-β1 for functional assays

Immune cells for functional experiments were isolated, seeded in 24-well plates at a density of 1-5 million of PBL or NK cells per ml of RPMI medium, treated for 72 h with 5 µM cortisol or 5 ng/ml TGF-β1 or both and then used for functional experiments.

2.2.6 Determination of cell viability using WST-1 assay (Roche)

The WST-1 assay is colorimetric assay designed to measure the relative metabolic activity of cells in culture. The assay is based on the reduction of the tetrazolium salt WST-1 into a colored dye by mitochondrial dehydrogenase enzymes. The soluble formazan salt is released into the medium. Within a certain time period, the reaction produces a color change which is directly proportional to the amount of mitochondrial dehydrogenase. As a result, the assay actually measures the net metabolic activity of cells which is a surrogate parameter for cell viability. From the cells that were left untreated or treated cells with cortisol or TGF-β1 or both, 10^4 cells were seeded in 100 µl medium per well in a 96-well plate. Thereafter, 10 µl per well of WST1-reagent was added, then incubated for further 4 h at 37°C and absorbance was measured in the photometer at 450 nm (correction wavelength 620 nm).

2.2.7 Lysis assay with K562-fLuc target cells (Brown et al., 2005)

To assess cytotoxicity, polyclonal NK cells were prepared, treated or not with cortisol or TGF-β1 or both for 72 h and used between day 8 and day 10 as cytotoxic effector cells against K562 targets that had been stably transfected with a firefly luciferase plasmid. These K562 fLuc target cells were seeded into opaque white 96-well plates, and NK cells were added in triplicates at the indicated E (ffector): T (arget) ratios. After addition of cell-permeable D-luciferin (PJK) at 0.14 mg/ml, the cells were further incubated and luminescence was detected at various time points. The ATP-dependent conversion of luciferin occurs only in viable luciferase-transfected cells and is thus directly proportional to cell viability.

2.2.7.1 Restimulation experiment with cortisol or TGF-β treated NK cells

On day 8 of the preparation, polyclonal NK cells were treated or not with cortisol or TGF-β1 or both for 72 h. Then, the NK cells were washed 2 times with fresh RPMI medium to remove the residues of cortisol or/and TGF-β1 before the differently pre-treated groups of washed cells were each divided again into 3 groups. First, cells with fresh medium only served as control group. In the second group, cells were co-cultured in fresh medium with MACSiBeads loaded with NKp46 and CD2 antibodies, using 5 µl (5×10^5 loaded MACSiBeads) per 10^6 NK cells. In the third group, cells were seeded in fresh medium onto plates that had been coated with antibodies against NKG2D and 2B4. Thereafter, all groups were incubated for further 48 h and then used as effector cells in a killing assay against K562-fLuc target cells.

2.2.8 Protein biochemical methods

2.2.8.1 Preparation of cell lysates from tumour cells and tissues

Five million tumour cells were centrifuged for 5 min at 450 x g at 4°C, then the cell pellet was washed with 100 µl of PBS to which protease and phosphates inhibitors (2 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µg/ml PMSF, 50 mM NaF, 200 µM $NaVO_5$ and 1 x phospho-stop, respectively) had been added. Then, 70 µl of lysis buffer-P containing protease and phosphatase inhibitors were added to each sample and the cells were lysed on ice for 20 min. Thereafter, the protein lysates were centrifuged for 15 min at 13,000 ×g at 4°C and the supernatant was transferred into new caps. Subsequently, the total amount of protein was determined in this cell lysate. Tumour tissues that had been stored at -80°C were first minced with mortar and pestle under liquid N_2 until they became a fine powder. Then, protein lysates were obtained using the same protocol as from tumour cells.

2.2.8.2 Determination of the total amount of protein by the Bradford method

To determine the total protein concentration in a cell lysate, the Bradford assay was performed. This is a colorimetric assay which is based on the property of the Coomassie Brilliant Blue dye to form complexes with proteins, thereby leading to a photometrically detectable colour shift. First, 50 µl of distilled water was loaded in every well of a 96-well plate, then 1 µl of protein lysate or Protein standard (HSA) was loaded in triplicate. For the standard row, protein concentrations of 1, 2, 4, 6, 8, 10 and 12 µg/ml were used. Thereafter,

Rotiquant Bradford reagent was diluted by 1:3.75 with distilled water, and then 200 µl of this solution were added to each well. After 20 min at room temperature, total protein concentration was determined in an ELISA reader by measuring the absorbance at 595 nm.

2.2.8.3 Immunoblotting

SDS- Polyacrylamide gels (containing between 8% and 15% of polyacrylamide) were prepared using a casting frame. For each sample, 20 µg of total protein lysate were mixed with the appropriate volume of reducing 5x Protein loading buffer and denatured for 5 min at 95°C, then chilled on ice for 3 min. The gel chambers were filled with running buffer, 20 µg of denatured proteins were loaded into each pocket of the polyacrylamide gel and 8-10 µl of a prestained protein standard were used to determine the size of the other loaded proteins. The SDS-PAGE run was carried out at 90 V for 20 min to collect the protein in the stacking gel, then a further 100 min at 130 V to separate the proteins in the running gel. The proteins were electro- blotted on a PVDF membrane at 145 V for 85 min using transfer buffer. The quality of the transferred protein was checked with Ponceau S staining. Afterwards, the membrane was washed with distilled water for 5 min and de-stained with PBS-T buffer for 5 min. Unspecific binding was minimized by incubating the membrane in blocking buffer for 2 h at RT, thereafter the primary antibody was incubated at 4°C overnight. Typical antibody concentrations ranged from 0.5-2 µg/ml. After incubation with the primary antibody diluted in the blocking buffer, the membrane was washed three times for 10 min with PBS-T, then incubated with HRP-coupled secondary antibody diluted in the blocking buffer (1:5,000) and washed again with PBS-T three times for 10 min. The HRP activity was detected using a home-made chemiluminescence detection system consisting of 1 ml solution A (50 mg Luminol in 200 ml 0,1 M Tris-HCl pH 6.8), 100 µl solution B (11 mg para-hydroxycoumarinic acid in 10 ml of DMSO) and 0.3 µl H_2O_2. These were mixed and then incubated on the membrane for 2 min, chemiluminescence was detected using X-ray films that were developed with developer solution (KODAK Developer) for 3 min and fixed for 1 min with fixative solution (KODAK Fixer). For re-probing, membranes were stripped with stripping buffer (0.2 M Glycin, 0,5 M NaCl, pH 2.8) for 10 min, followed by neutralization with neutralizing buffer (25 mM Tris 193 mM Glycin, 0,5% SDS pH 8.8) for 10 min. After further blocking, the membrane could then be incubated again with another antibody, e.g. a loading control like β-actin.

2.2.9 Methods for analysis and manipulation of gene expression

2.2.9.1 Isolation of RNA from tumour and immune cells

For isolation of RNA from immune cells or tumour cells, $5x10^6$ cells were pelleted and treated with 1 ml TriFast reagent at room temperature for 5 min. Then, per 1ml TriFast 0.2 ml of chloroform were added to the sample, followed by shaking for 15 sec and further incubation for 5 min at room temperature. Thereafter, the mixture was centrifuged for 15 min at 12,000 ×g at 4°C and the upper aqueous phase containing the RNA was carefully transferred into a fresh vial. To precipitate the RNA, an equal amount of isopropanol was added to the aqueous phase and incubated for 10 min on ice, then centrifuged for 15 min at 12,000 ×g at 4°C. The RNA pellet was washed twice with 75% EtOH. To precipitate the RNA after each wash, it was centrifuged for 5 min at 7,500 xg at 4°C. Then, the RNA pellet was dried in the air. The RNA pellet was finally dissolved in 30 µl of double deionized (dd) H_2O.

2.2.9.2 Determination of RNA concentration

The RNA concentration was determined using a spectrophotometer. The RNA was diluted 1:100 in (dd) H_2O; the absorbance was measured at 260 and 280 nm against (dd) H2O water control. The concentration was calculated using the formula: C [µg / ml] = $OD_{260\ nm}$ x dilution factor x 40

The quality of the isolated RNA was estimated by dividing the value obtained for the absorbance at 260 nm by the absorbance measured at 280 nm. Ideally, the ratio $OD_{260\ nm}/OD_{280\ nm}$ should be 1.8.

2.2.9.3 Synthesis of cDNA from isolated RNA (BioRad)

iScriptTM cDNA Synthesis Kit was used for synthesis of cDNA from isolated RNA. This kit consists of a modified MMLV (Mouse Moloney Leukemia Virus)-derived reverse transcriptase with RNase H endonuclease activity, an RNase inhibitor and a blend of oligo(dT) and random hexamer primers. Oligo(dT) primers bind to the polyA-tail contained in all cellular mRNAs while random hexamers can bind within any given RNA sequence. After primer binding at 25°C, reverse transcription occurred during 30 min at 42°C in a PCR thermocycler.

Table (2): Composition of cDNA synthesis reaction.

Component	Volume
5x iScript Reaction Mix	4 µl
iScript reverse transcriptase	1 µL
RNA template	0.5 ug
Nuclease-free water	to 20 µl

Table (3): cDNA synthesis program steps.

Temperature	Incubation period
25°C	5 min
42°C	30 min
85°C	5 min

2.2.9.4 Reverse transcriptase Polymerase Chain Reaction (PCR)

To qualitatively measure the expression level of mRNA transcripts, cDNA was used as template in PCR reactions. PCR reactions were performed using the Crimson longAmp Polymerase Kit and a PCR thermocycler.

Table (4): Composition of the PCR reaction.

Component	Volume
5 x Crimson LongAmp Taq Buffer	5 µl
10 mM dNTP	0.75 µl
100 µM forward primer	1 µl of 1:10 diluted primer
100 µM reverse primer	1 µl of 1:10 diluted primer
cDNA	1 µl
(dd) H_20	to 25 µl
AmpliTaq Polymerase	0.5 µl

Table (5): The program steps for PCR Reaction

Temperature	Incubation time
94°C	3 min
94°C	30 sec 35 cycles
Primer-dependent annealing temperature (e.g. 60 °C)	30 sec 35 cycles
72°C	45 sec 35 cycles
72°C	10 min
4°C	Hold

2.2.9.5 Polymerase Chain Reaction (PCR) For Mouse genotyping

2.2.9.5.1 Isolation of genomic DNA from mouse ear punches (Truett et al., 2000)

Mouse ear punches were treated with 100 µl of a mixture containing 25 mM NaOH and 0.2 mM EDTA (pH 8) and placed for 60 min at 95°C in a heating block. Then, 100 µl of 40 mM Tris HCl (pH 5.5) were added and the sample was spun down at 4,000 rpm for 3 min. The supernatant which contains the genomic DNA was transferred to a fresh vial. For genotyping, about 1µl from this supernatant was used.

2.2.9.5.1.1 Kras genotyping

Table (6): Composition of the PCR reaction for Kras genotyping.

Component	Stock	Volume
Crimson LongAmp Taq Buffer	5x	5µl
dNTP	10 mM	0.75µl
dt5-Primer	100µM → 1:2.5	0.6µl
Uni-Primer	100µM → 1:2.5	0.4µl
SD5-Primer	100µM → 1:2.5	0.2µl

gDNA	1µl
(dd) H_20	to 25µl
Crimson LongAmp Taq DNA Polymerase	0.5µl

Table (7): The program steps for Kras PCR Reaction.

Temperature	Incubation time
94°C	3 min
94°C	30 sec 35 cycles
64°C	1 min 30 sec 35 cycles
72°C	1 min 35 cycles
72°C	5 min
4°C	Hold

PCR product size:

- wt 500bp
- loxP-Stop-loxP-Kras^{G12D} 550bp

2.2.9.5.1.2 Pten genotyping

Table (8): Composition of the PCR reaction for Pten genotyping.

Component	Stock	Volume
Crimson LongAmp Taq Buffer	5x	5µl
dNTP	10 mM	0.75µl
Pten 6637-F Primer	100µM	0.25µl
Pten 6925-R Primer	100µM	0.25µl
Pten 7319-R Primer	100µM	0.25µl
gDNA		1µl
(dd) H_20		to 25µl

Crimson LongAmp Taq DNA Polymerase	0.5µl

Table (9): The program steps for Pten PCR Reaction.

Temperature	Incubation time
94°C	3 min
94°C	45 sec 40 cycles
60°C	30 sec 40 cycles
72°C	1 min 40 cycles
72°C	5 min
4°C	Hold

PCR product size:

- wt 500bp
- loxP-Pten-loxP 650bp
- del ex5 300bp

2.2.9.5.2 DNA gel electrophoresis

For analysis of PCR products, 1% agarose gels were prepared in 1 x TAE buffer stained with 3 µl of GelRed. Thereafter, 10-20 µl of PCR product mixed with 2 µl 6 x DNA loading buffer were loaded and the gels were run in 0.5 x TAE buffer for 1 h at 90 V. To determine the size of the PCR product, a standard 100 bp DNA ladder was loaded on the gel. The separated DNA bands were visualized by UV light and documented with a digital camera.

2.2.9.5.3 Semi-quantitative real time PCR

For relative quantification of mRNA transcript levels, real time PCR (qRT-PCR) was applied. To this aim, the genes of interest were amplified from the synthesized cDNA using the ABsolute Blue QPCR SYBR Green low Rox mix kit for qRT-PCR. The purity of the PCR products was assessed via the dissociation curve. The samples were used in duplicates and only used when the deviation of C_t-values was ≤ 0.5 cycles. (In a real time PCR assay, the C_t (cycle threshold) value is defined as the number of cycles required for the accumulating

fluorescent signal to cross the threshold, i.e. to exceed background level. C_t levels are inversely proportional to the amount of target nucleic acid in the sample which means that a low C_t level corresponds to a relatively large amount of target nucleic acid in the sample.) The relative expression of mRNA was quantified relative to the expression of the 18S rRNA and with the expression of untreated control or healthy control and calculated according to the $\Delta\Delta C_t$ method, using the formula $rI = 2^{[(C_t \text{ gene of interest, investigated sample} - C_t \text{ 18S investigated sample}) - (C_t \text{ gene of interest, reference sample} - C_t \text{ 18S reference sample})]}$.

Table (10): Composition of the qRT-PCR reaction.

Component	Volume
2 x ABsolute Blue QPCR SYBR Green low Rox mix	7.5 µl
Forward primer (1:90 diluted in (dd) H_20)	1 µl
Reverse primer (1:90 diluted in (dd) H_20)	1 µl
Water for injection	0.5 µl
cDNA (1:20 in water for injection)	5 µl

Table (11): The program steps for qRT-PCR reaction were performed in the ABI TaqMan 7500.

Temperature	Incubation time
50°C	2 min
95°C	15 min
95°C	15 sec 40 cycles
60°C	1 min 40 cycles
95°C	15 sec dissociation curve
60°C	1 min
95°C	15 sec

2.2.10 Immunohistochemical staining

All human tissue specimens were provided by the tumour bank of the University Hospital Würzburg. Mouse tissue specimens were collected from the animal facility in the Institute for Virology and Immunobiology (Würzburg University, Germany). Frozen tissue samples were cut at 4 µm, fixed in an ice-cold acetone/methanol mixture (1:1) for 5 min and rehydrated in TBS buffer for 15 min. The non-specific binding to tissue antigens was blocked with 10% normal goat serum in TBS buffer for 30 min, and endogenous peroxidases were left to react with 3% H_2O_2. After washing with TBS buffer for 10 min, the slides were incubated overnight with diluted primary antibody. Following 3 washes with TBS buffer for 5 min, the slides were incubated for 1 h with a secondary biotinylated antibody, then washed again 3 times with TBS buffer for 5 min followed by HRP-conjugated streptavidin for 30 min - 1 h. Stainings were developed with diaminobenzidine for 15 min. Nuclei were counterstained with haematoxylin. Stained sections were then dehydrated by washing in graded ethanol and embedded in Vitro-Clud.

2.2.11 Cortisol levels in serum and ascitic fluid

2.2.11.1 Cortisol luminescent immunoassay (LIA)

Cortisol concentrations were determined in serum and ascitic fluid of ovarian cancer patients by a luminescent immunoassay (LIA) (IBL, Hamburg, Germany) that had originally developed for analysis of cortisol concentration in saliva (cortisol saliva, IBL, Hamburg). This luminescent immunoassay is based on the competition principle: An unknown amount of antigen present in the sample competes with a fixed amount of enzyme-labeled antigen for binding to a limiting amount of plate-bound antibody. After incubation, the wells are washed to remove unbound labeled and unlabeled antigen. Then, luminescence substrate solution is added and luminescence is quantified in a luminometer, the measured luminescence intensity is inversely proportional to the amount of the antigen in the sample. Cortisol concentrations can thus be determined according to a standard curve. Since cortisol shows a strong diurnal regulation, all samples were obtained at similar times in the morning. Serum samples from healthy donors were used as reference healthy control and all measurements were performed in triplicate. Results were further validated using reference samples provided in the kit.

2.2.12 Isolation of bone marrow for adoptive transfer

To investigate the effect of specific mutations in the immune system, hematopoietic stem-cell transfer has become a standard technique. To this aim, host hematopoietic cells are first killed by ionizing radiation, and then hematopoietic system is replaced by transfusion of donor bone marrow or purified hematopoietic stem cells. The resulting individuals are termed irradiation bone marrow chimeras.

Bone marrow was flushed under sterile conditions from the medullary cavities of tibiae and femurs of CD45.1 (Ly5.1), $GR^{flox/flox}$ and Lck-Cre $GR^{flox/flox}$ mice, using RPMI1640 medium and a 21-gauge needle. (A T cell-specific knockout mutant of the GR was generated by a collaboration partner (Wüst et al., 2008) by introducing two loxP recombination sites around exon 3 of the GR (Nr3c1) and cross-breeding the $GR^{flox/flox}$ mice with mice expressing a Cre transgene under the control of the proximal lck promoter (Lck-Cre). Due to activation of the Lck-promoter during T cell development, the resulting Lck-Cre $GR^{flox/flox}$ mice possess ly GR-deficient T cells.) A single cell suspension was obtained by passing the whole bone marrow through a 70 µm cell strainer. Then, cells were washed and counted and $CD90.2^-$ hematopoietic stem cells were isolated by magnetically-activated cell sorting (MACS) technology (Miltenyi).

2.2.12.1 Isolation of the $CD90.2^-$ hematopoietic stem cells by Magnetically-activated cell sorting (MACS) technology

CD90 is a differentiation marker that is expressed on hematopoietic stem cells. Depending on the respective mouse strain, 2 different isoforms CD90.1 or CD90.2 can be expressed which can be used to trace adoptively transferred cells. In our setting, however, the two different isoforms of CD45 (Ly5.1) were used for tracing while CD90.2 represented a differentiations marker. For magnetic depletion of differentiated T cells, the CD90.2 $(Thy1.2)^+$ T cells are first magnetically labeled with CD90.2 MicroBeads. Then, the cell suspension is loaded onto a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled $CD90.2^+$ cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of $CD90.2^+$ cells. The $CD90.2^-$ hematopoietic stem cells are immature stem cells which can become tolerized upon adoptive transfer and thus do not mount a graft-versus-host reaction in the recipient mice.

2.2.12.2 Adoptive transfer of hematopoietic stem cells from Lck-Cre $GR^{flox/flox}$ mice

To investigate the immunomodulatory role of endogenous glucocorticoids in ovarian cancer *in vivo*, 129 S_4/SvJae-C57BL/6JloxP-*Stop*-loxP-K-$ras^{G12D/+}$$Pten^{loxP/loxP}$-mice were irradiated with 11.5 gray divided into two doses (6 Gy and 5.5 Gy at an interval of 4 hours) to completely eradicate the recipient animals' hematopoietic stem cells. Within 24 hours, immunological reconstitution was performed by a single intra-venous injection of $CD90.2^-$ hematopoietic stem cells obtained from bone marrow of Lck-Cre $GR^{loxP/loxP}$ mice (Glucocorticoid receptor deficient T cells) and $GR^{loxP/loxP}$ mice (reconstituted control). As additional control, mice were irradiated without bone marrow reconstitution. These animals died within 10-12 days from the effects of anemia. Since all used strains of mice endogenously express the CD45.2 allele, another control group was reconstituted with bone marrow from transgenic C57Bl/6-CD45.1 mice. This enabled the identification of adoptively transferred immune cells via an antibody raised against the CD45.1 allele. Thus, the reconstitution efficiency could be determined. Once the immune system of these irradiated mice had been reconstituted with glucocorticoid-insensitive T cells, ovarian carcinoma was subsequently induced by injection of the adenoviral Cre-recombinase into the ovarian bursa. The comparison between mice that had been reconstituted with glucocorticoid-responsive or glucocorticoid-resistant T cells, respectively, provides information on the role of endogenous glucocorticoids on T cells and on general anti-tumour immune responses in ovarian carcinoma.

2.2.13 Statistics

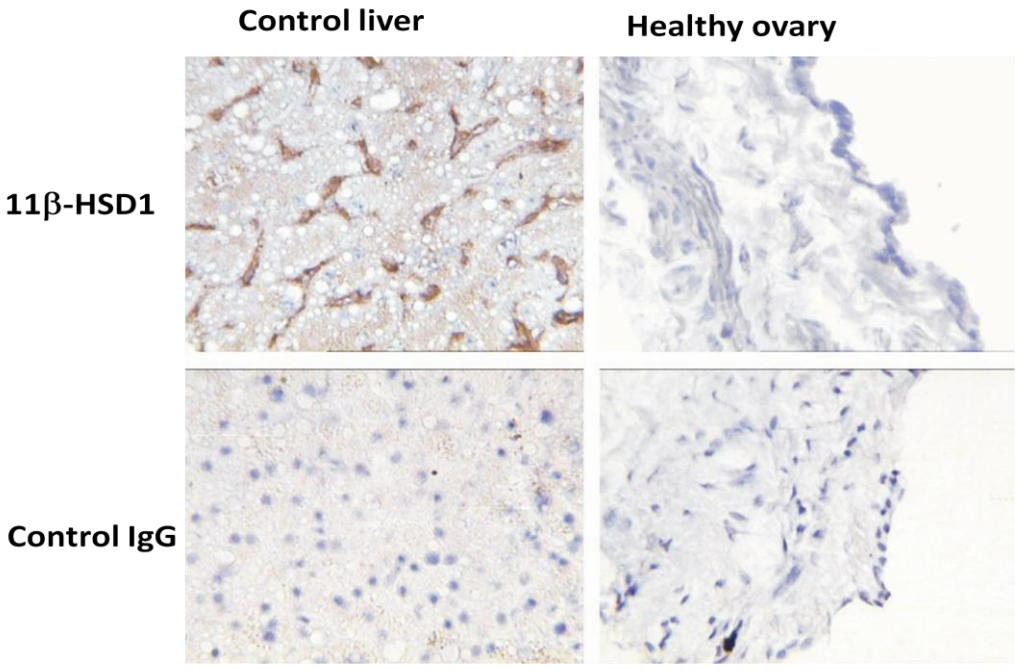
Experiments were performed at least three times with similar results. P values were determined by unpaired student t-test using Graphpad Prism 4. For flow cytometry data, SDs are indicated as calculated from the raw data by Summit software (BECKMAN COULTER).

3 Results

3.1 Expression of 11β-HSD1 in human ovarian cancer

3.1.1 Expression patterns of 11β-HSD1/2 in cryopreserved human ovarian cancer samples

The expression of the 11β-HSD1/2 was initially examined by immunohistochemistry on cryopreserved human tumour samples. Sections of liver (11β-HSD1) and placenta (11β-HSD2) were stained as positive controls; healthy tissues were stained as negative controls. The results showed a high expression of the isoenzyme 11β-HSD1 in 18/20 ovarian carcinomas (15 of the serous papillary, 5 of the endometroid subtype) but not in the 5 investigated healthy ovaries.



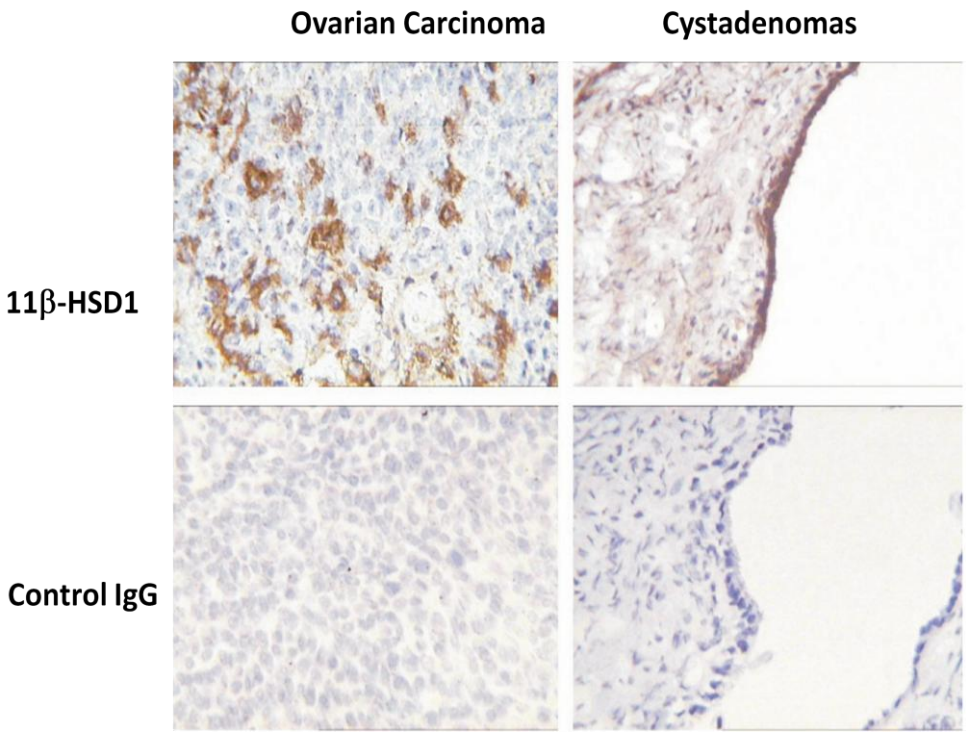


Figure (15): Overexpression of 11β-HSD1 in human ovarian cancer tissues. Cryosections from liver (n = 2), healthy ovary (n = 5), cystadenomas (n=3) and ovarian cancers (n=20) were fixed in ice-cold acetone, rehydrated for 1 h with Tris-buffered saline, blocked with 10 % normal goat serum and incubated overnight at 4°C with rabbit anti-human 11β-HSD1 antibody, followed by secondary HRP-labelled anti-rabbit–IgG. Stainings were developed using diaminobenzidine. (Pictures by courtesy of Dr. Claudia Heidbrink).

Expression of the 11β-HSD2 was hardly detectable in the ovarian cancer samples.

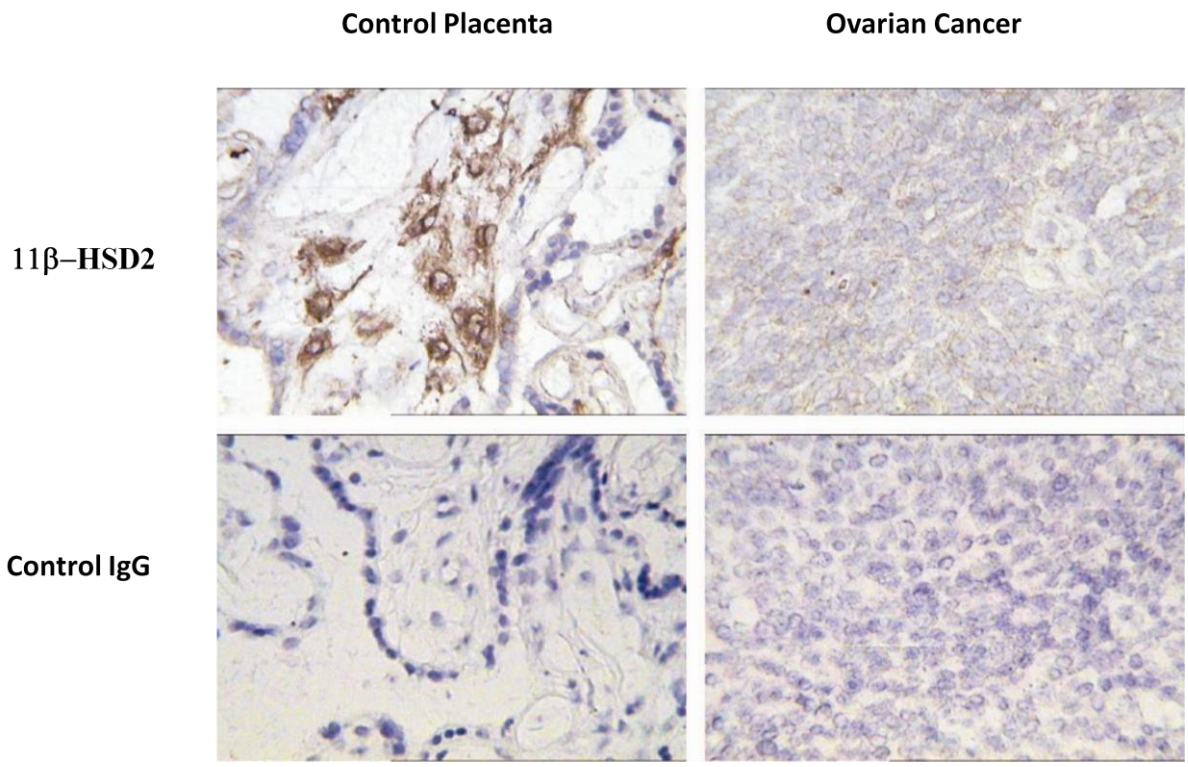


Figure (16): 11β-HSD2 was hardly detected in human ovarian cancer tissues. Cryosections from human placenta (n = 2) and ovarian cancers (n = 10) were stained as in the previous Figure (15), using a rabbit anti-human 11β-HSD2 antibody. (Pictures by courtesy of Dr. Claudia Heidbrink).

3.1.2 Expression of 11β-HSD1 in fresh human ovarian tumour tissues and EpCAM-positive ovarian cancer cell lines

Total RNA was isolated from fresh human ovarian tumour tissue and ovarian cancer cell lines, transcribed into cDNA and the relative mRNA expression levels were determined by SybrGreen-based (semi-) quantitative real-time PCR (qRT)-PCR.

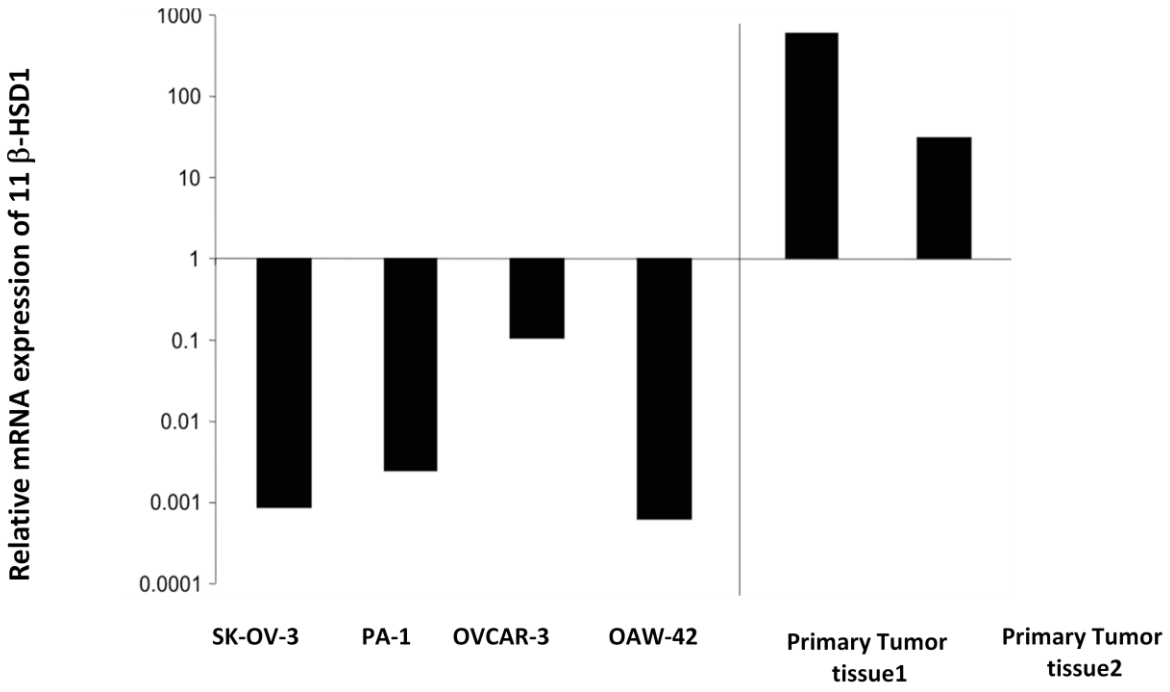
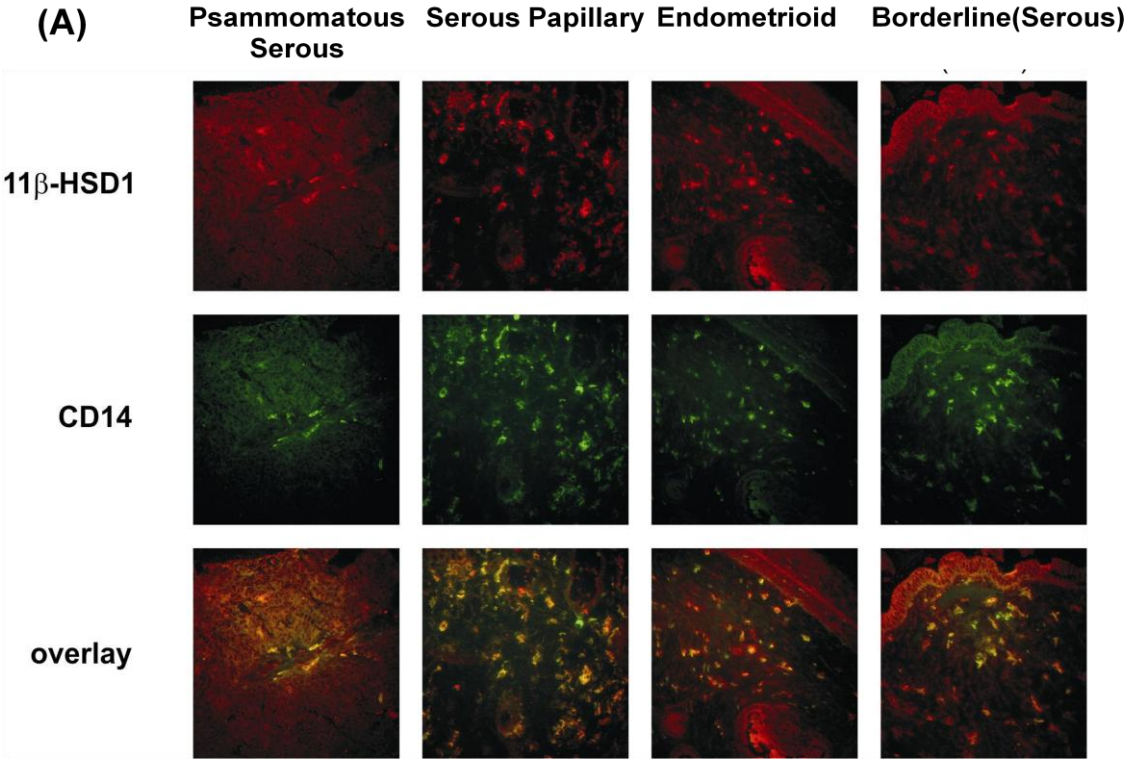
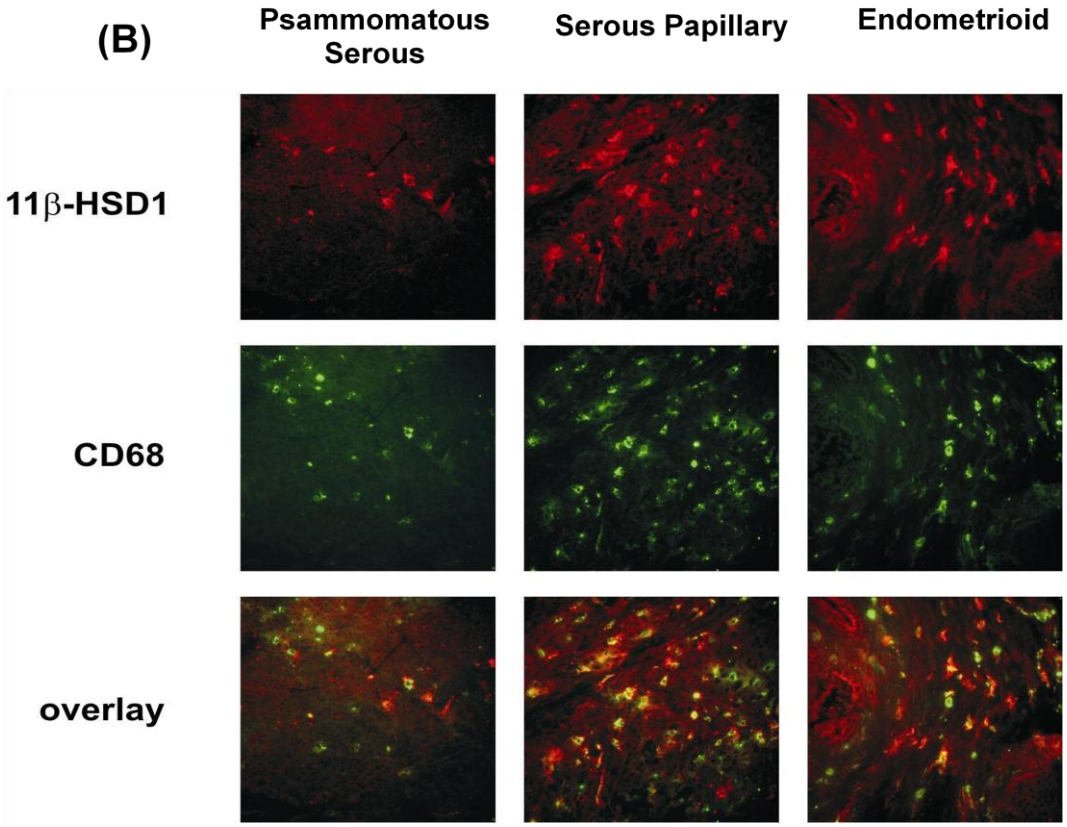


Figure (17): Overexpression of 11β-HSD1 in human ovarian cancer tissues. RNA was isolated from ovarian cancer cell lines and primary tumour tissue and reverse-transcribed into cDNA. The transcriptional expression of 11β-HSD1 was determined by SybrGreen-based quantitative PCR. Data were collected by Dr. Claudia Heidbrink and analyzed according to $\Delta\Delta C_t$-method.

3.1.3 Expression of 11β-HSD1 in human OvCA tissues with CD14, CD68, CD85 and EpCAM

The cellular attribution of 11β-HSD1 expression in human ovarian cancer samples was examined by immunofluorescent double staining on cryosections from serous papillary, psammomatous serous and endometrioid ovarian carcinomas. This revealed a co-localization of 11β-HSD1 with CD14, CD68, and CD85, but not with the tumour marker EpCAM.




(B)
Psammomatous Serous
Serous Papillary
Endometrioid
11β-HSD1
CD68
overlay

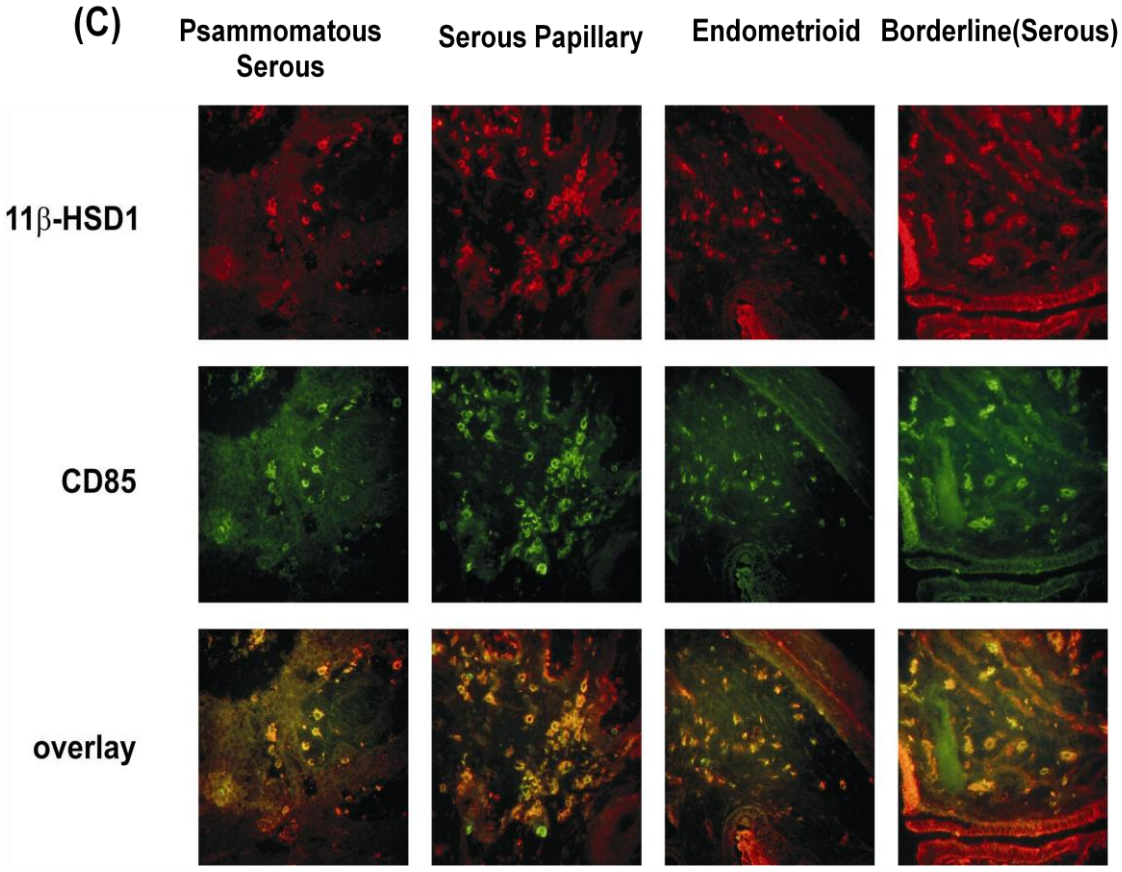

(C)
Psammomatous Serous
Serous Papillary
Endometrioid
Borderline(Serous)
11β-HSD1
CD85
overlay

Figure (18): Immunofluorescent double staining of 11β-HSD1 with CD14, CD68, and CD85 in OvCA. Cryosections from 5 serous papillary, 5 psammomatous serous, 3 endometrioid ovarian carcinomas and 2 borderline tumours were stained with rabbit anti-human 11β-HSD1 antibody, antibodies against human CD14 (A), CD68 (B) and CD85(C). Immunofluorescent double staining was visualized by conjugated anti-Rabbit Alexa555 antibody and conjugated anti-mouse Alexa488 antibody. (Pictures by courtesy of Dr. Claudia Heidbrink).

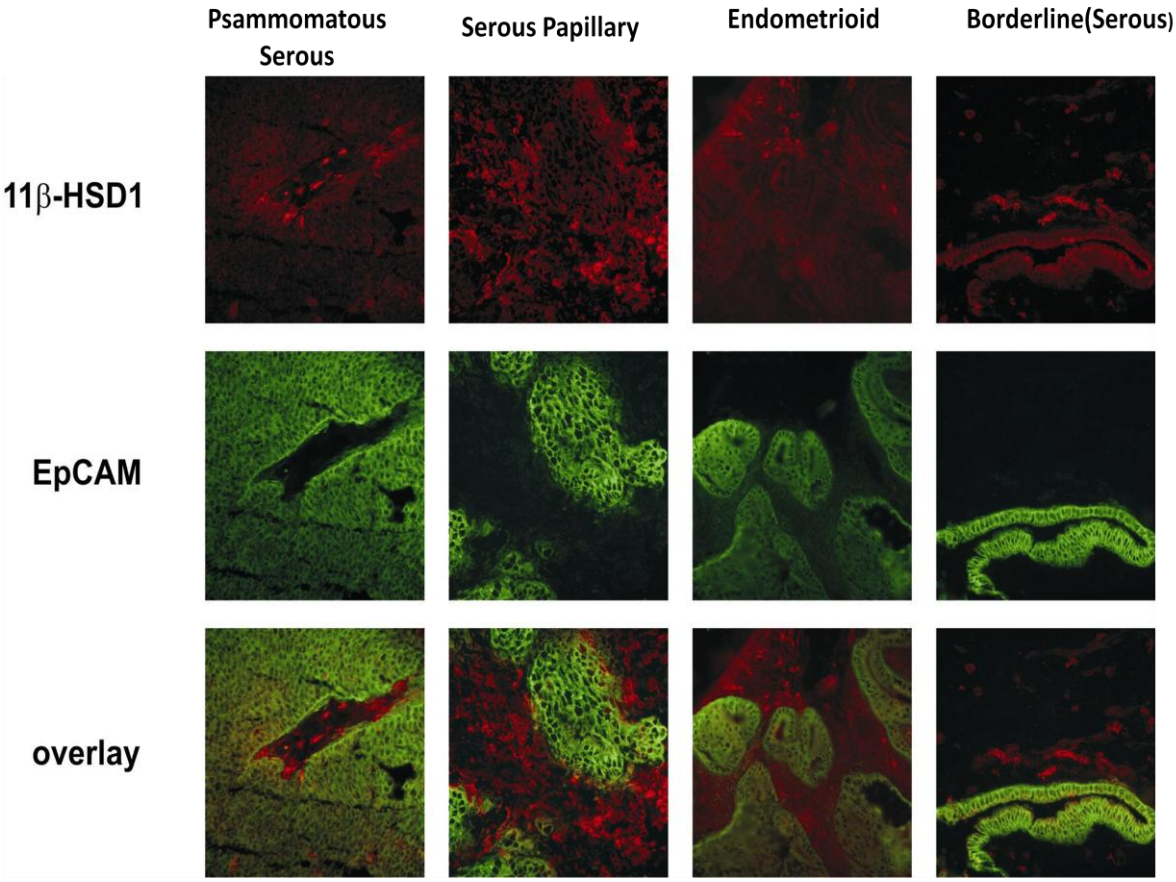


Figure (19): Immunofluorescent double staining of 11β-HSD1 with EpCAM in OvCA. Cryosections from 5 serous-papillary, 5 psammomatus-serous, 3 endometrioid ovarian carcinomas and 2 borderline tumours were stained with rabbit anti-human 11β-HSD1 antibody, conjugated mouse anti-human EpCAM-FITC antibody. 11β-HSD1 staining was visualized by conjugated anti-Rabbit Alexa555 antibody. (Pictures by courtesy of Dr. Claudia Heidbrink).

3.2 Cortisol concentrations in serum, tumour exudates and ascites of OvCA patients

Over-expression of 11β-HSD1 in ovarian cancer was associated with elevated endogenous glucocorticoid levels. Cortisol levels in serum, tumour exudates and ascites of ovarian cancer patients were determined by luminescent immunoassays (LIA) which are based on competition between endogenous cortisol and the labelled glucocorticoid contained in the kit. The result showed elevated cortisol levels in serum, ascites and tissue exudates from ovarian cancer patients as compared to serum from healthy controls. It was strictly ensured that patients had not previously been treated with glucocorticoids of any kind for at least three months.

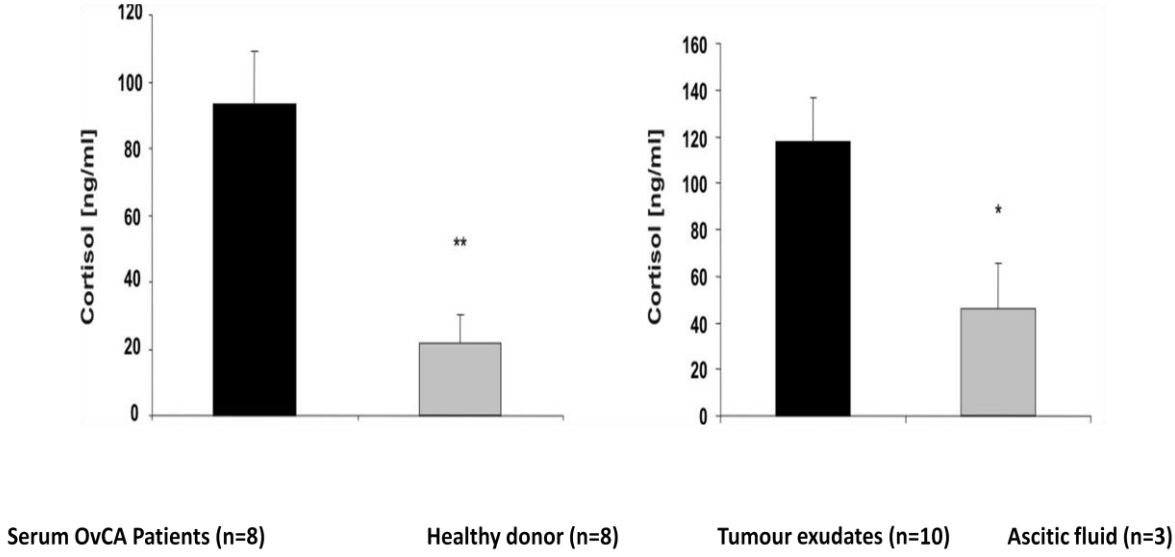


Figure (20): High levels of cortisol in serum, tumour exudates and ascites of OvCA patients. Using a luminescence-based immunoassay which was originally developed for analysis of cortisol in saliva, the morning cortisol concentrations were determined in serum, tumour exudates and ascites of ovarian cancer patients against a standard curve (correlation coefficient p = 0.998). Serum samples from healthy control persons were collected at the same time and used as reference controls. All measurements were performed in triplicate. (Data obtained from Dr. Claudia Heidbrink.)

3.3 Expression of GR in human OvCA-tissues and and ovarian cancer cell lines

Protein lysates were isolated from normal ovaries, ovarian cancer (solid-papillary and endometrioid) and ovarian cancer cell lines. Glucocorticoid receptor (GR) expression on protein levels was determined by Western blot. GR was expressed in 17 out of 18 OvCA tissue samples and in 9/9 OvCA cell lines. The result revealed that cortisol may have direct effects on ovarian cancer cells.

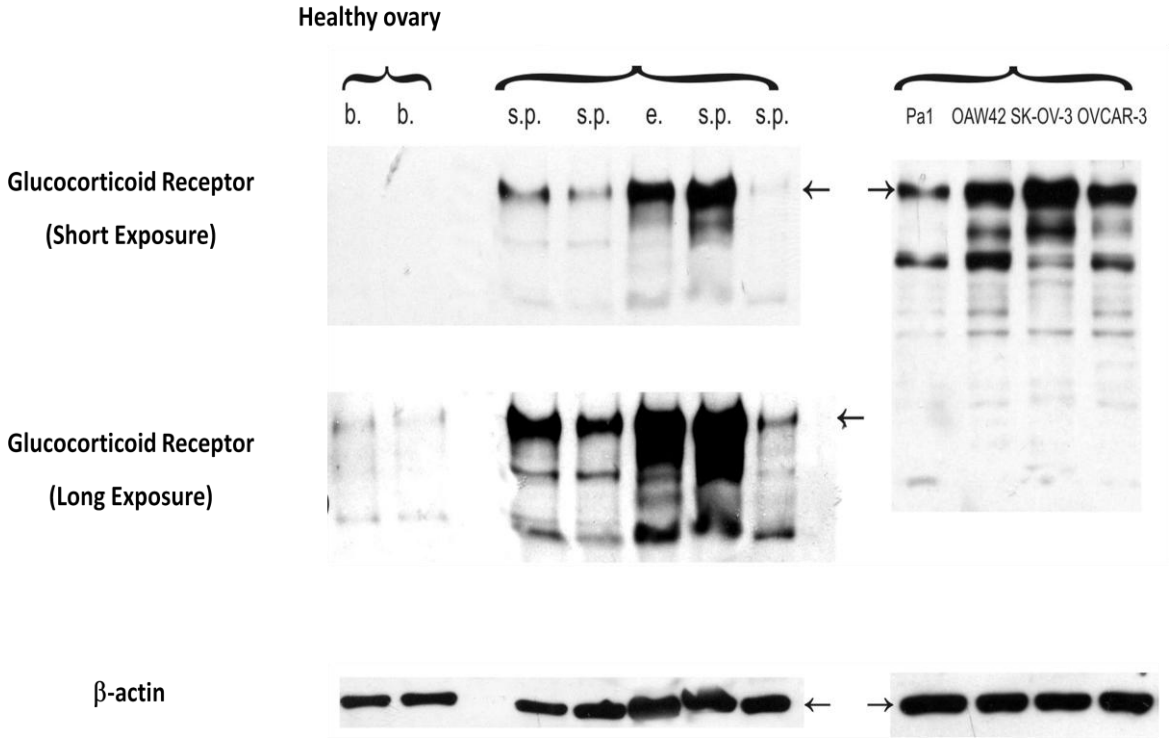


Figure (21): GR expression in normal ovaries, ovarian cancer and ovarian cancer cell lines. Expression of GR (95 kDa) was determined by Western blotting in samples from normal ovaries, ovarian cancer (sp = solid-papillary, e =endometrioid) and in ovarian cancer cell lines. Protein lysates were separated on a 10% SDS-polyacrylamide gel loaded with 20 µg of total protein per lane, rabbit anti-human glucocorticoid receptor antibody was used, followed with HRP-conjugated secondary antibody. The same blot was stripped and neutralized for further detection of the endogenous loading control β-actin. (Analysis performed by Dr. Claudia Heidbrink).

3.4 Viability and metabolic activity of the OvCA cell lines after treatment with cortisol

Viability and metabolic activity of cortisol-treated OvCA cell lines were determined using the WST assay. For this purpose, the chromogenic tetrazolium salt WST was added to the cells and absorbance was measured 4 h later in an ELISA reader. The color reaction is based on the reduction of WST which can only occur in metabolically active (i.e. viable) cells. The result showed that treatment of OvCA cell lines with exogenous cortisol (up to 100 µM) had no effect on their proliferation and viability.

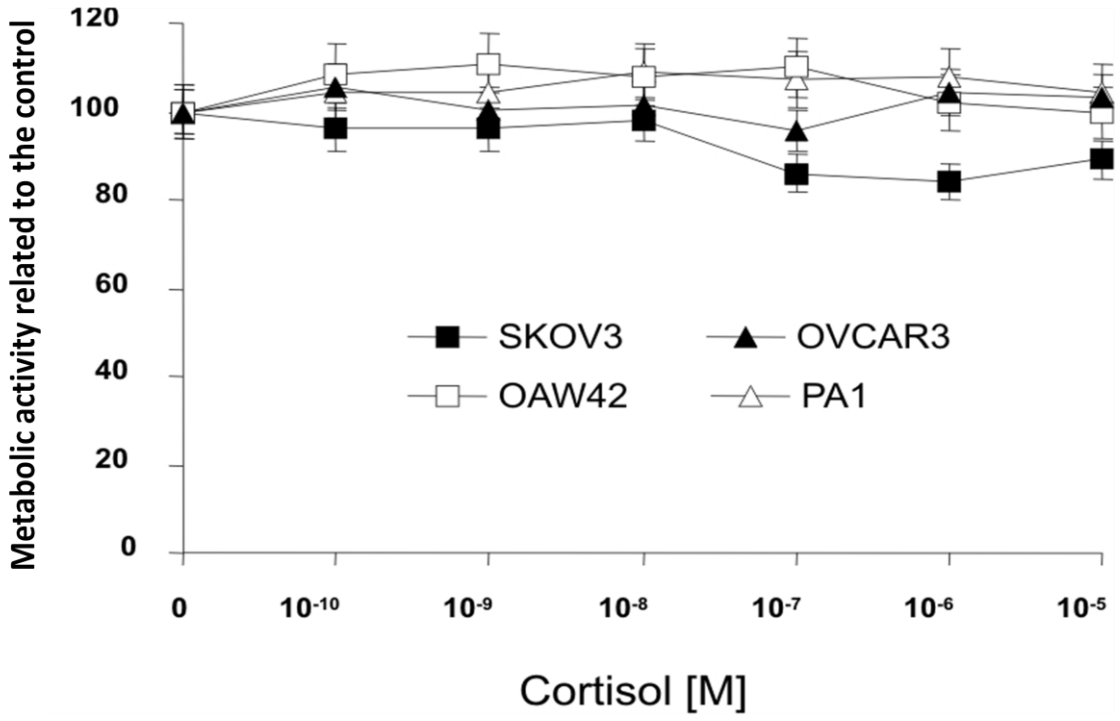


Figure (22): WST turnover of cortisol treated OvCA cell lines. Ovarian cancer cells were seeded in 96 well plates and treated the next day with different concentrations of cortisol. To determine the metabolic activity of treated and untreated cells, the tetrazolium salt WST-1 was added after 46 h of incubation with cortisol. The colorimetric reaction of the tetrazolium salt WST was measured 4 h after addition to the cells in an ELISA reader at OD 450 nm (correction wavelength: 620 nm). Under these experimental conditions, the measured absorbance is proportional to the number of living cells. The intense brown color reaction is based on the reduction of WST which can only occur in metabolically active (= viable) cells. (Data kindly provided by Dr. Claudia Heidbrink).

3.5 Mouse ovarian carcinoma model

3.5.1 Spontaneous mouse model for ovarian cancer

A spontaneous mouse model for endometrioid ovarian cancer used to investigate the functional immunomodulatory role of endogenous GCs in ovarian cancer. For this model, an adenovirally encoded Cre-recombinase (AdCre) was injected into the ovarian bursa of 129 S_4/SvJae-C57BL/6JloxP-*Stop*-loxP-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice to induce the deletion of the PTEN tumour suppressor gene and the activation of the latent oncogenic K-ras^{G12D} (Dinulescu et al., 2006). Due to the deletion of PTEN and the activation of K-ras, the mice developed ovarian cancer of the endometrioid subtype which became visible within 6-7 weeks. Later, the mice also developed peritoneal metastases as evidenced by the formation of ascites in the abdominal cavity.

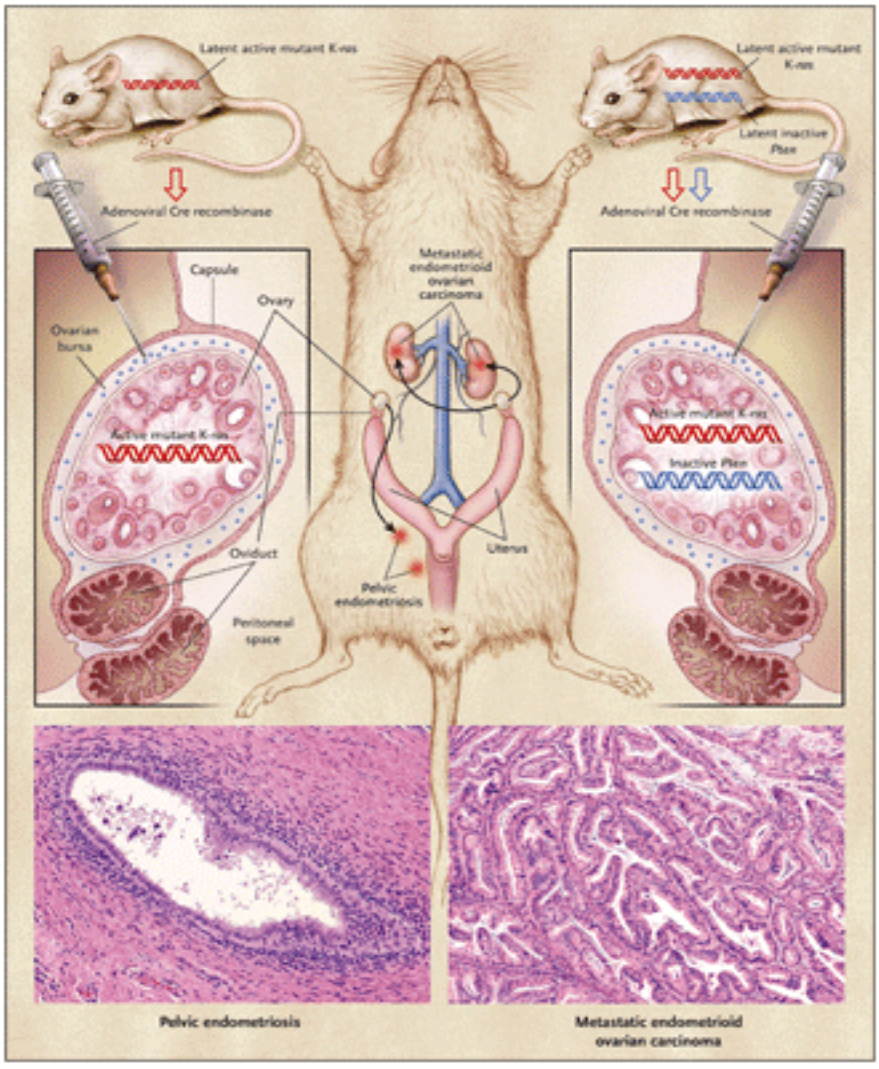
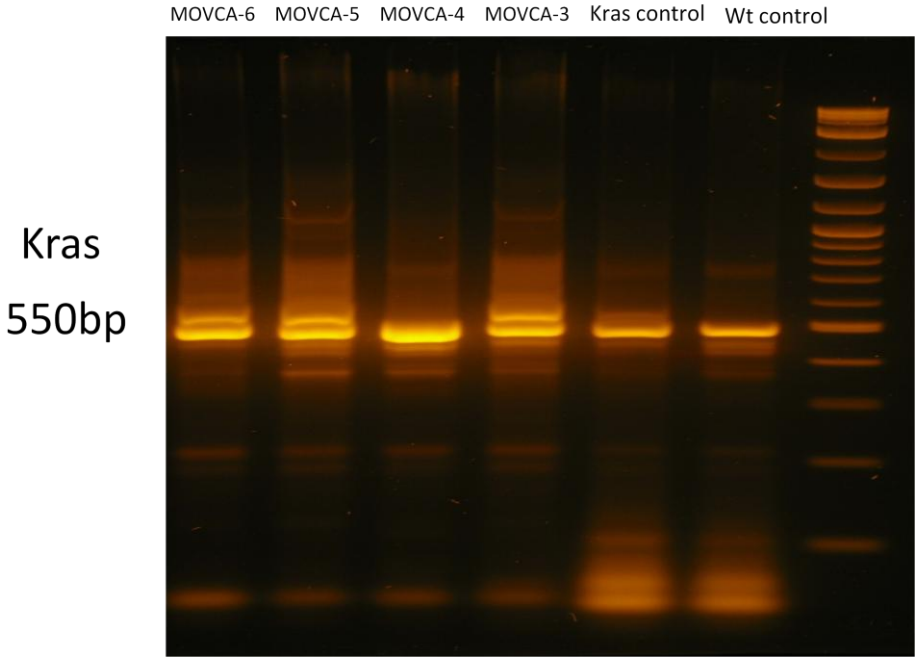


Figure (23): Making a Mouse Model of Gynecologic Disease (Jeff Boyd, 2005; Dinulescu et al., 2006). Dinulescu et al. genetically engineered mice to carry latent alleles of active mutant K-ras (left side) or active mutant K-ras and inactive Pten (right side). Injection of an adenoviral Cre recombinase construct into the ovarian bursa led to tissue-specific expression of active mutant K-ras, resulting in pelvic endometriosis, or tissue-specific expression of active mutant K-ras and inactivation of Pten, resulting in metastatic endometrioid ovarian carcinoma.

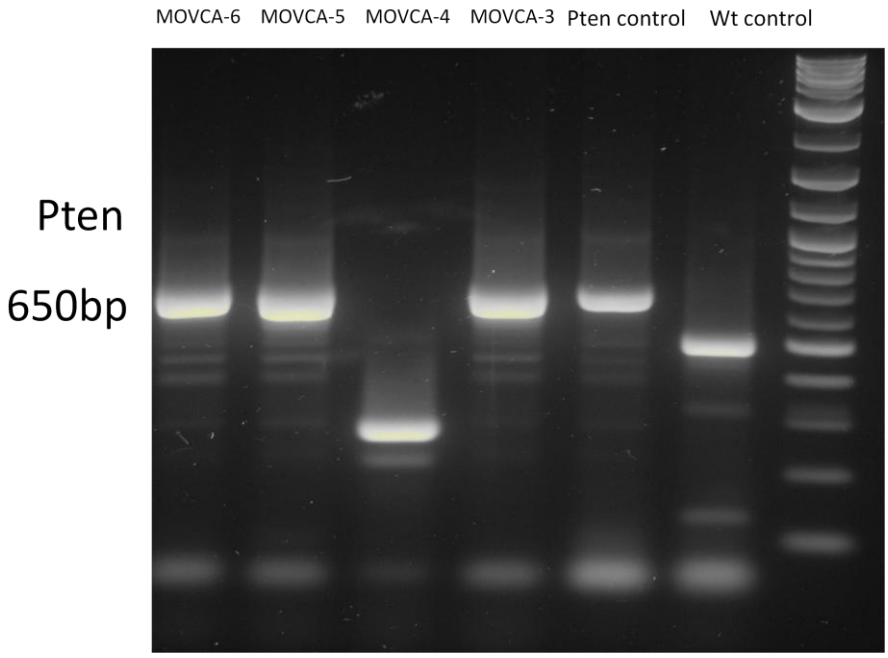
3.5.2 Generation of cell lines from mouse ovarian tumour

I managed to generate four mouse ovarian cancer cell lines (MOVCA -3, -4, -5 and -6) originate from our spontaneous mouse model for endometrioid ovarian cancer (Dinulescu et al., 2006) by digesting mouse ovarian cancer tissues via dispase and a single cell suspension was obtained by passing the digested mixture through a 70 µm cell strainer. Thereafter, cells were repeatedly passaged *in vitro.* Then, cell lines were obtained by using limiting dilution method in order to clone individual cells per well. All cell lines were EpCAM positive. Moreover, three of these cell lines are expressing *K-ras* and *Pten* whereas one cell line is having *K-ras* and lacking *Pten.* In future, these mouse ovarian cancer cell lines could help for further investigation of molecular and immune interactions in ovarian cancer development.

A



B


MOVCA-6
MOVCA-5
MOVCA-4
MOVCA-3
Pten control
Wt control
Pten
650bp

C

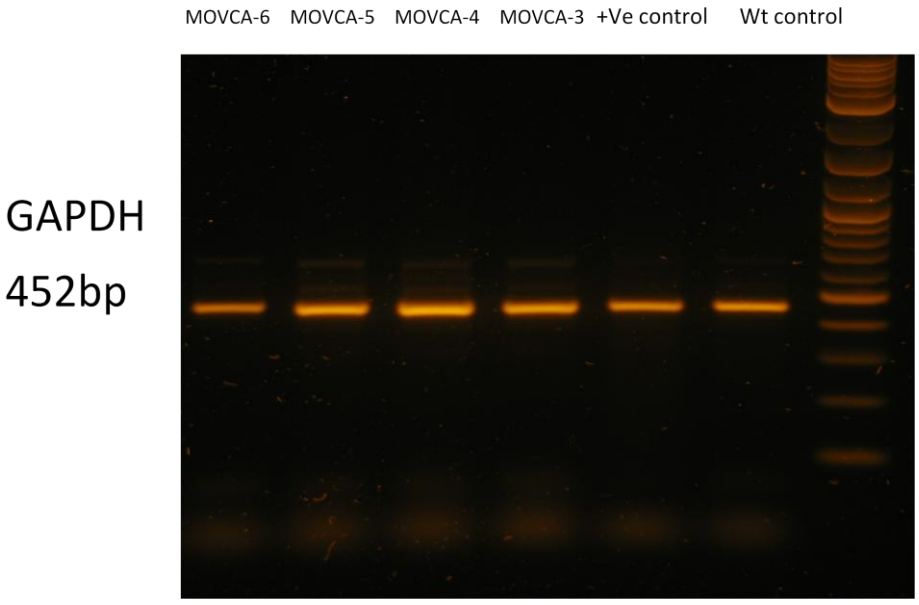

MOVCA-6
MOVCA-5
MOVCA-4
MOVCA-3
+Ve control
Wt control
GAPDH
452bp

Figure (24): Expression of *Kras*, *Pten* in mouse ovarian cancer cell lines. RNA was isolated from murine ovarian cancer cell lines or *Kras*/*Pten* ovarian cancer tissue as positive control and Wt as negative control and reverse-transcribed into cDNA. *Kras* (A) and *Pten* (B) expression levels were determined using RT-PCR. GAPDH was amplified as loading control (C).

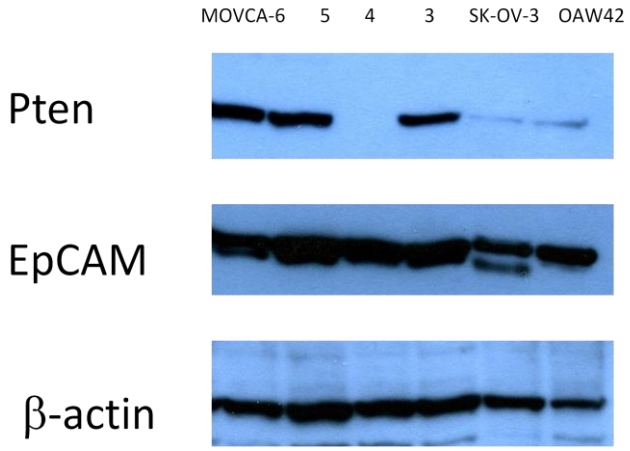


Figure (25): GR expression in normal ovaries, ovarian cancer and ovarian cancer cell lines. Expression of *Pten* (54 kDa) and EpCAM (34 kDa) were determined by Western blotting in samples from murine ovarian cancer cell lines and SK-OV-3 or OAW42 as positive controls. Protein lysates were separated on a 10% SDS-polyacrylamide gel loaded with 20 µg of total protein per lane, rabbit anti-mouse Pten antibody was used, followed with HRP-conjugated secondary antibody. For EpCAM biotin rat anti-mouse antibody was used, followed with followed by Streptavidin-HRP. The same blot was stripped and neutralized for further detection of the endogenous loading control β-actin.

3.6 Expression of 11β-HSD1 in murine ovarian cancer

To check whether 11β-HSD1 and 11β-HSD2 expression levels in the inducible murine ovarian cancer model reflect those previously observed in the human disease, we performed immunohistochemical staining on cryopreserved mouse ovarian tumour material. Healthy ovarian tissues from non-induced littermates were stained as negative controls.

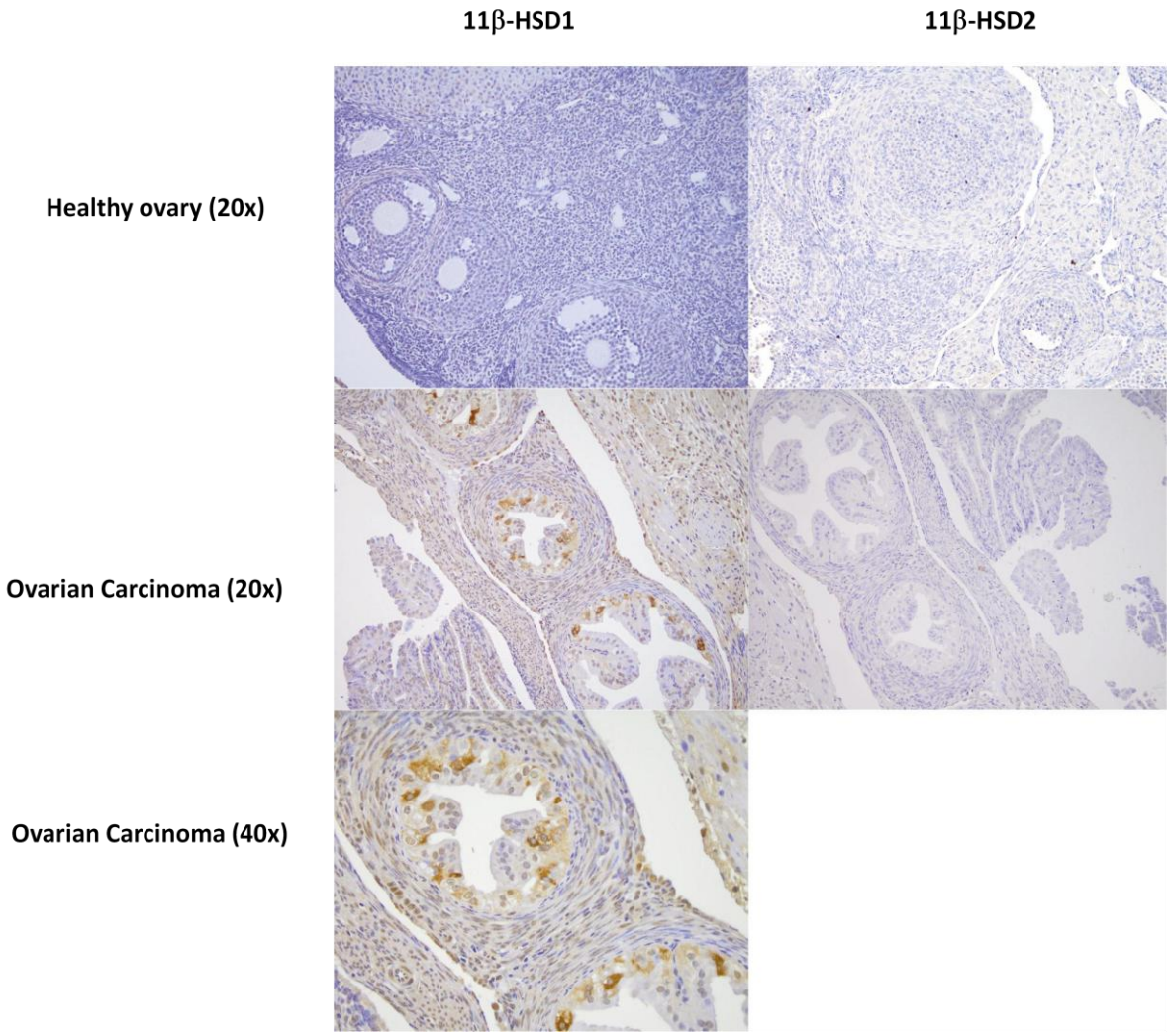


Figure (26): Overexpression of 11β-HSD1 in mouse ovarian cancer tissue. The 129S4/SvJae-C57BL/6J loxP-*Stop*-loxP-*K-ras*^{G12D}/*Pten*$^{loxP/loxP}$ mice were sacrificed 8 weeks after intra-bursal injection of adenoviral Cre which leads to the induction of an endometrioid ovarian cancer. Tumours (middle and bottom) and contralateral normal ovaries (above) were frozen in liquid nitrogen. Sections of ovarian cancer tissues and normal ovaries tissues were stained with rabbit anti-mouse 11β-HSD1 (left) and sheep anti-mouse 11β-HSD2 (right). (Pictures by courtesy of Dr. Sebastian Häusler).

3.7 Adoptive transfer of hematopoietic stem cells from GR-deficient mice

The intratumoral expression of 11β-HSD1 in the inducible mouse model for ovarian cancer opened up the possibility to investigate whether glucocorticoid-insensitive immune cells could mount a superior immune response against the tumour. To this aim, bone marrow chimeras were created. Thus, recipient mice harboring the Pten$^{loxP/loxP}$ and loxP-*Stop*-loxP-kras^{G12D} alleles were first irradiated and then adoptively transferred with CD90.2^{-} hematopoietic stem cells isolated from the bone marrow of either Lck-Cre GR$^{loxP/loxP}$mice (T cell specific glucocorticoid receptor knock-out mice) or GR$^{loxP/loxP}$ mice. (Due to the absence of Cre-recombinase, these mice display similar GR expression levels as the wild-type controls.) Bone-marrow derived CD90.2^{-} hematopoietic stem cells from CD45.1 (Ly5.1) mice (n=2) served as additional control to monitor the reconstitution of the immune system with the adoptively transferred cells. All irradiated recipient mice were injected with 2x10^{6} CD90.2^{-} cells into the tail vein with the exception of 2 mice that did not receive bone marrow transfer and thus died from anemia after 8-10 days. After the adoptively transferred cells had been allowed to engraft for 30 days, the mice that had received CD45.1 cells were sacrificed and immunological reconstitution was confirmed by FACS analysis of CD45.2 (host) and CD45.1 (transferred) cells from the spleen. After reconstitution, ovarian cancer was induced by injection of the adenoviral Cre-recombinase into the ovarian bursa and tumour development was observed. The survival percentage of these mice was plotted in a Kaplan-Meyer curve.

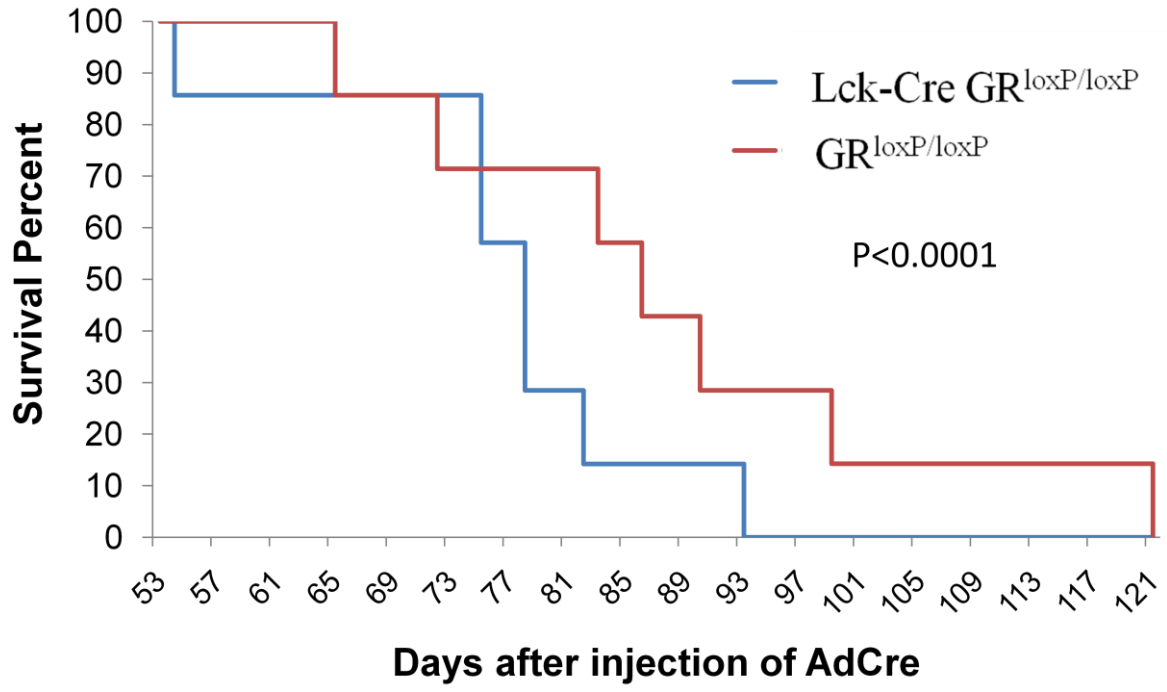


Figure (27): Adoptive transfer of control and GR-deficient immune cells into 129S4/SvJae-C57BL/6J loxP-*Stop*-loxP-*K-ras*^{G12D}/*Pten*$^{loxP/loxP}$ recipient mice followed by induction of ovarian cancer. A Kaplan-Meyer curve was plotted for recipient mice that had received Lck-Cre GR$^{oxP/loxP}$ (n=5) or GR $^{loxP/loxP}$ control cells (n=7) before Cre-mediated induction of ovarian cancer. P value was determined by unpaired student t-test using Graphpad Prism 4.

Contrary to our hypothesis, we observed that mice with glucocorticoid-insensitive T cells show an even shorter survival than their littermates with a normal (though adoptively transferred) immune system. This raised the question whether T cells that were no longer inhibited by endogenous glucocorticoids might assume a phenotype that promotes rather than inhibits tumour growth. Such properties have been described for regulatory T cells. We thus checked tumours from both groups for the presence of tumour-infiltrating immune cells with particular attention on $T_{reg.}$

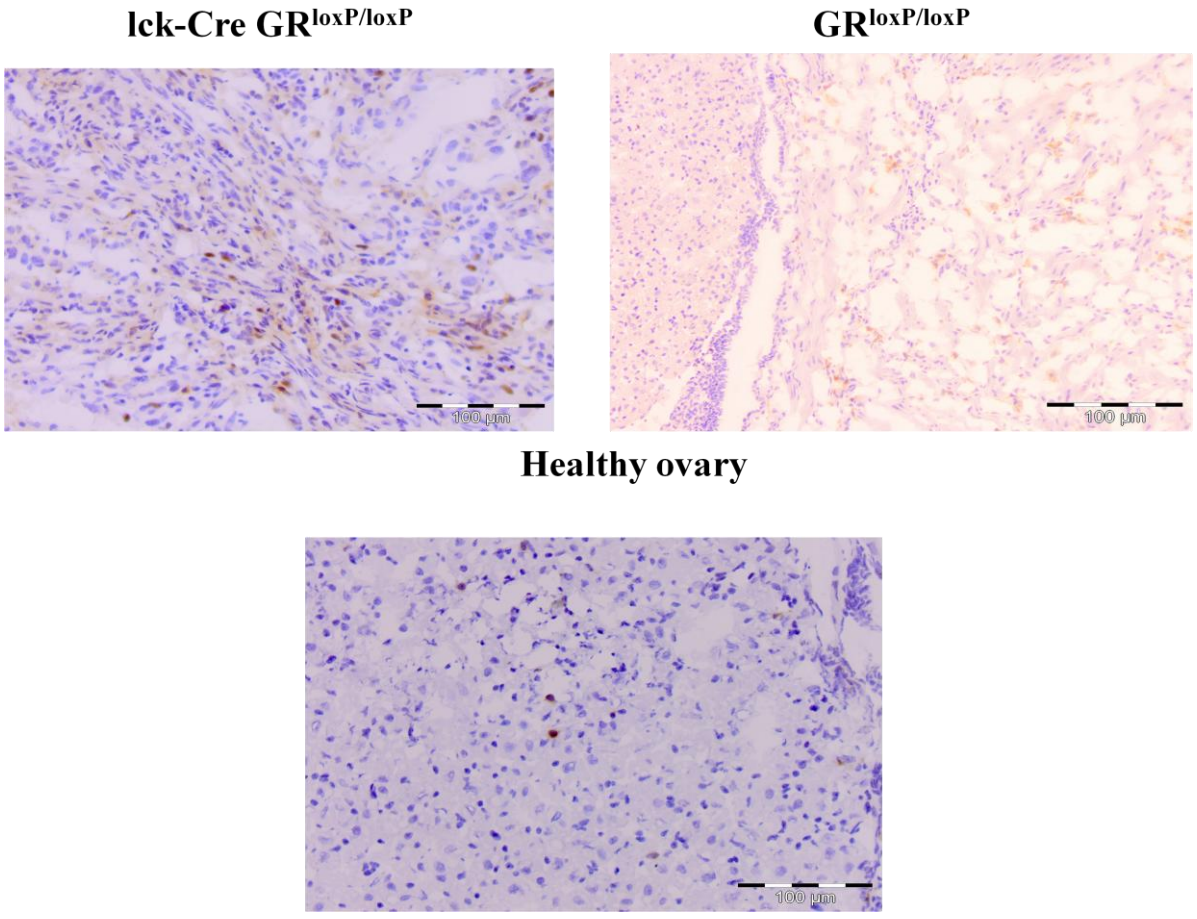


Figure (28): Expression of $CD4^+$ T cells in murine ovarian cancer tissues. Cryosections of ovarian cancer tissues from mice reconstituted with bone marrow obtained from Lck-Cre $GR^{loxP/loxP}$ (n=1) or $GR^{loxP/loxP}$ (n=1) control mice and healthy ovarian tissues (n=1) were fixed in ice-cold acetone, rehydrated for 1 h with Tris-buffered saline, blocked with 10 % normal goat serum and incubated overnight at 4 °C with rat anti-mouse $CD4^+$ antibody, followed by secondary HRP-labelled anti-rat–IgG. The stainings were developed with diaminobenzidine.

3.8 Expression of $FOXP3^+$ in murine ovarian tumour tissues

The expression of the T_{reg} master transcription factor $FOXP3^+$ was determined by immunohistochemistry on cryopreserved mice tumour samples. Sections of ovarian cancer tissues from mice reconstituted with bone marrow from Lck-Cre $GR^{loxP/loxP}$ or $GR^{loxP/loxP}$ control mice were stained for $FOXP3^+$; healthy ovarian tissues were stained as negative controls. The result showed that $FOXP3^+$ was highly expressed in the ovarian cancer from mice reconstituted with hematopoietic stem cells from Lck-Cre $GR^{loxP/loxP}$ donor mice but not in mice reconstituted with $GR^{loxP/loxP}$ control cells or healthy ovarian tissues. Thus, it may by hypothesized that abrogation of GR signaling in T cells improves T cell infiltration into the tumour tissue. However, T cells that have entered the tumour microenvironment tend to assume a tolerogenic phenotype. Accordingly, inhibition of endogenous glucocorticoids can even have a negative overall effect unless further immunosuppressive mechanisms (e.g. conditioning of the tumour microenvironment by TGF-β) are also addressed.

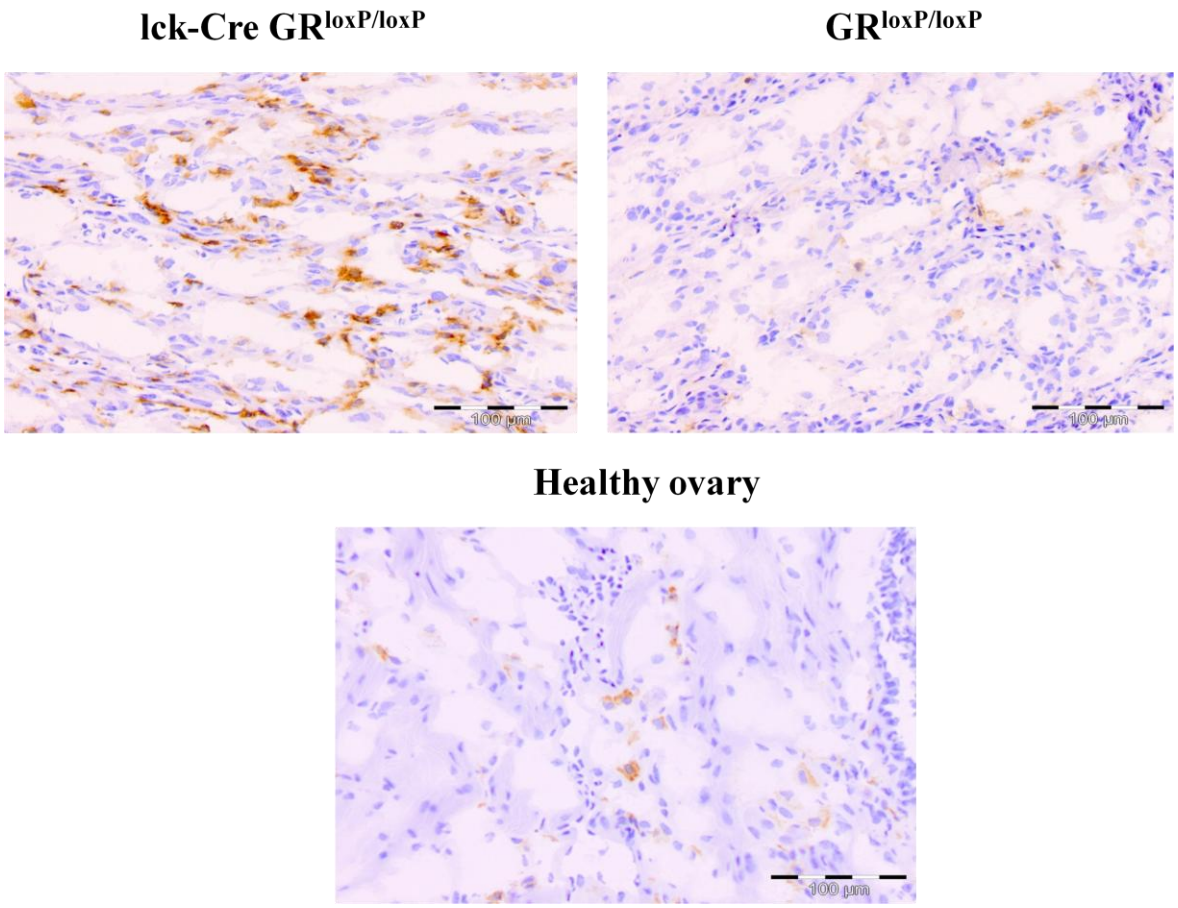


Figure (29): Expression of FOXP3$^+$in murine ovarian cancer tissues. Cryosections of ovarian cancer tissues from mice reconstituted with bone marrow obtained from Lck-Cre GR$^{loxP/loxP}$ (n=1) or GR$^{loxP/loxP}$ (n=1) control mice and healthy ovarian tissues (n=1) were fixed in ice-cold acetone, rehydrated for 1 h with Tris-buffered saline, blocked with 10 % normal goat serum and incubated overnight at 4°C with mouse anti-mouse FOXP3$^+$ antibody, followed by secondary HRP-labelled anti-mouse–IgG. The stainings were developed with diaminobenzidine.

3.9 Expression of DCIR2 in murine ovarian tumour tissues

CD8$^+$DEC-205/CD205$^+$ dendritic cells (DCs) may convert Foxp3$^-$ precursors into Foxp3$^+$ T_{reg} in a TGF-β independent manner. CD8$^-$DCIR2$^+$ DCs do, in contrast, promote the expansion of Foxp3$^+$ T_{reg} in the presence of TGF-β (Yamazaki et al., 2008). Since these CD8$^-$DCIR2$^+$ constitute the major fraction of inhibitory dendritic cells (DCs), we assessed expression of DCIR2 by immunohistochemistry on cryopreserved mice tumour samples. Sections of ovarian cancer tissue from mice reconstituted with bone marrow from Lck-Cre GR$^{loxP/loxP}$ or

$GR^{loxP/loxP}$ control mice were stained for DCIR2; healthy ovarian tissues were stained as negative controls. This revealed a greatly enhanced DCIR2 expression in ovarian cancer specimens from mice reconstituted with hematopoietic cells from Lck-Cre $GR^{loxP/loxP}$donors, but not in mice reconstituted with control ($GR^{loxP/loxP}$) bone marrow or healthy ovarian tissues. Considering that the only difference between mice reconstituted with Lck-Cre $GR^{loxP/loxP}$ and mice reconstituted with $GR^{loxP/loxP}$ cells is GR expression in T cells which then leads to different levels of T cell infiltration, it appears likely that changes in antigen-presenting cells occur in response to infiltrating T cells. Moreover, the high abundance of $CD8^{-}DCIR2^{+}$ DCs which can expand natural $Foxp3^{+}$ T_{reg} in a TGF-β dependent manner may explain the high numbers of $Foxp3^{+}$ T cells in this group and point towards a role for TGF-β.

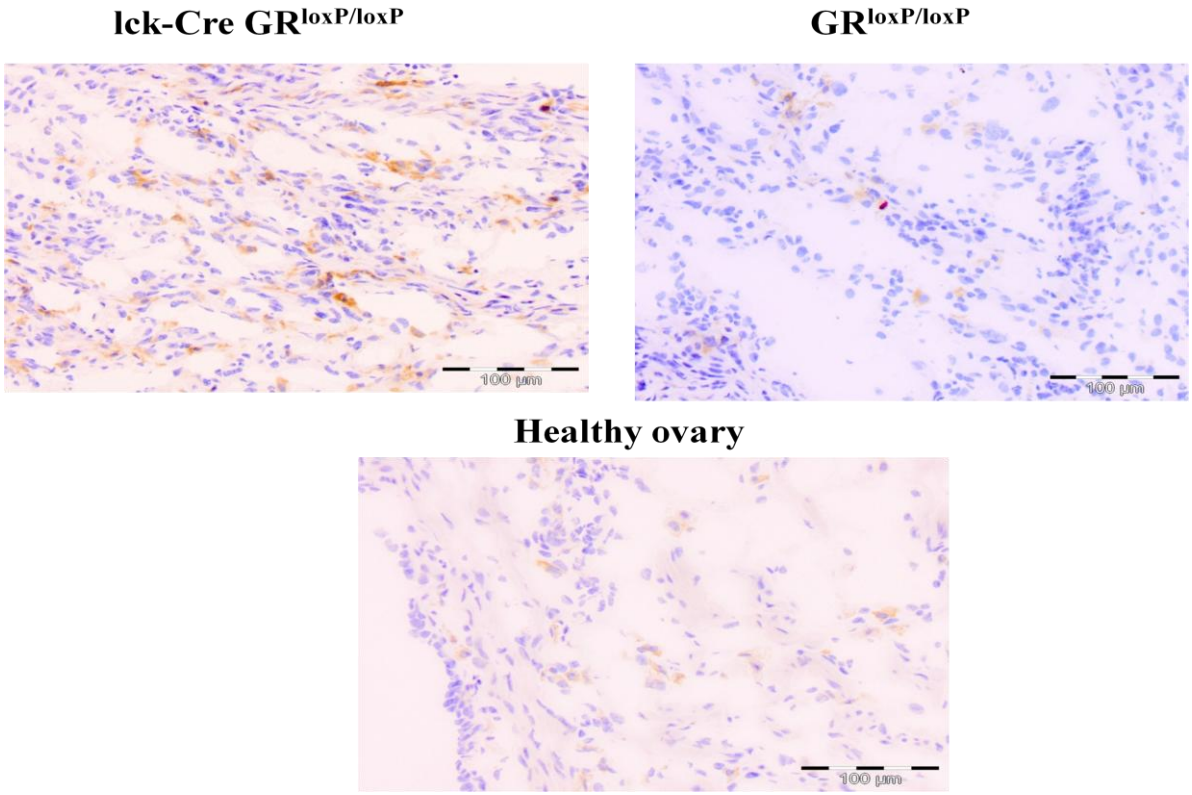


Figure (30): Expression of DCIR2 in mouse ovarian cancer tissues. Cryosections of ovarian cancer tissues from mice reconstituted with bone marrow obtained from Lck-Cre $GR^{loxP/loxP}$ (n=1) or $GR^{loxP/loxP}$ (n=1) control mice and healthy ovarian tissues (n=1) were fixed in ice-cold acetone, rehydrated for 1 h with Tris-buffered saline, blocked with 10 % normal goat serum and incubated overnight at 4 °C with biotin rat anti-mouse DCIR2 antibody, followed by Streptavidin-HRP. The stainings were developed using diaminobenzidine.

3.10 Expression of 11β-HSD1 in murine ovarian tumour tissues from bone marrow chimeras

Ovarian carcinomas grown in irradiated 129S4/SvJae-C57BL/6J loxP-*Stop*-loxP-*K-ras*^{G12D}/*Pten*$^{loxP/loxP}$ mice that had been reconstituted with bone marrow stem cells from lck-Cre GR$^{loxP/loxP}$, GR$^{loxP/loxP}$ and CD45.1 (Ly5.1) mice were explanted, RNA was extracted from the various tumours and from healthy ovaries of control mice, cDNA was prepared via reverse transcription and the expression of 11β-HSD1 was determined by reverse transcriptase polymerase chain reaction.

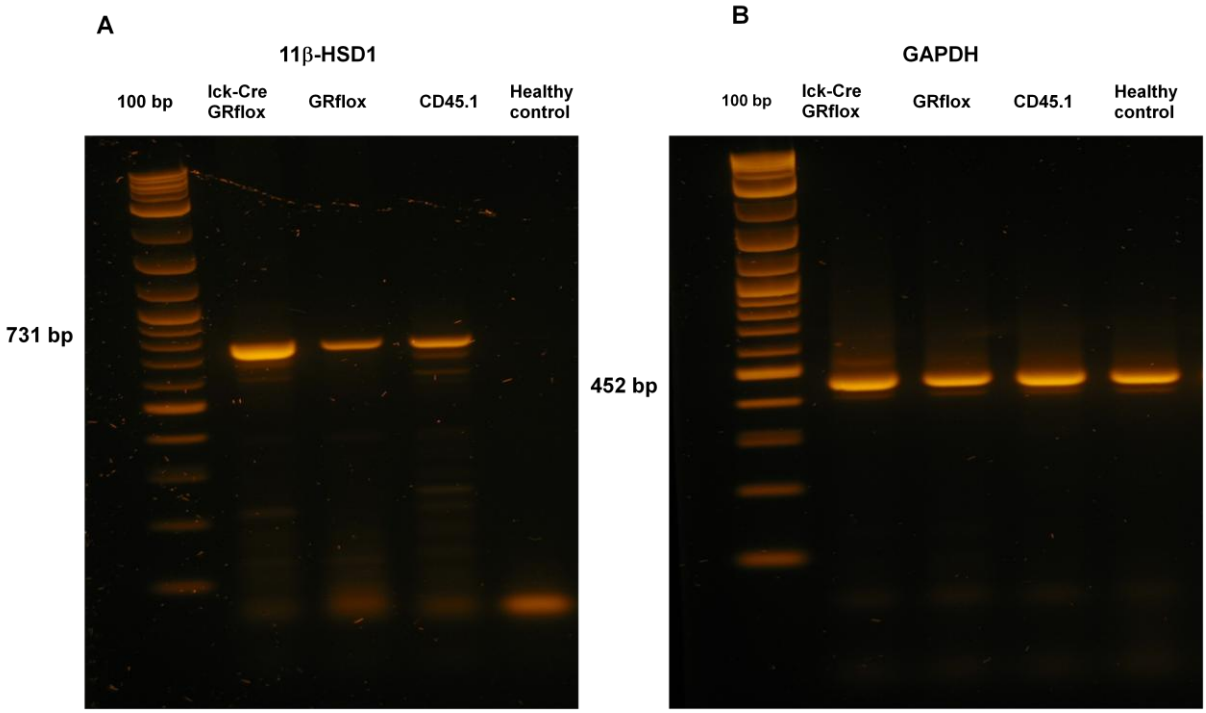


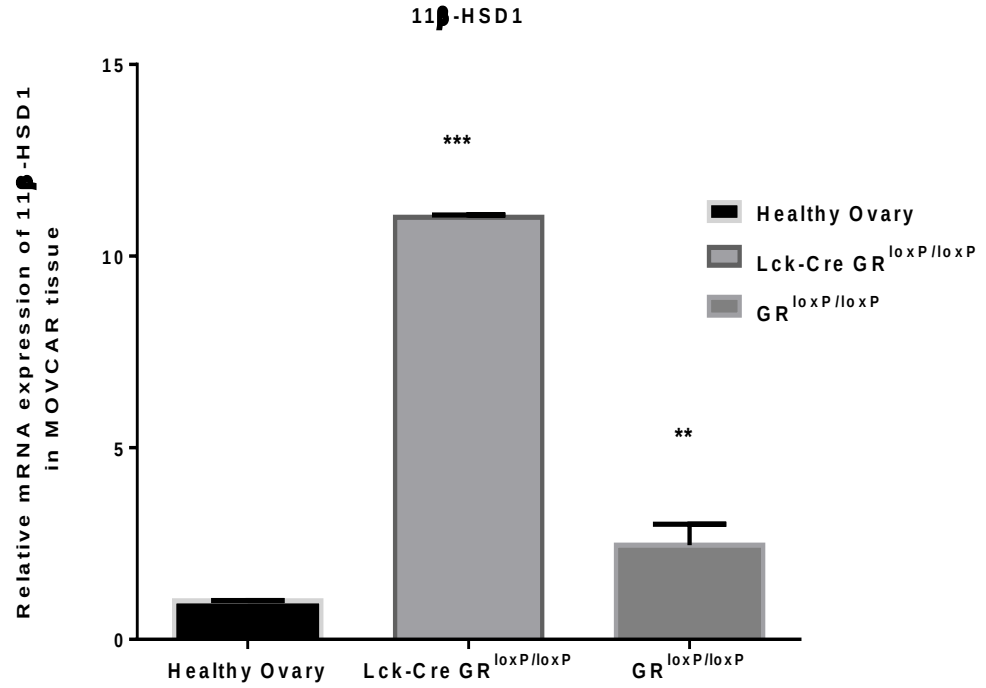
Figure (31): Expression of 11β-HSD1 in mice ovarian cancer tissues. RNA was isolated from murine ovarian cancer tissues of Lck-Cre GR$^{loxP/loxP}$ (n=2) or GR$^{loxP/loxP}$ (n=2) control mice or CD45.1 (n=2) control mice and healthy ovarian tissues (n=2) and reverse-transcribed into cDNA. 11β-HSD1 expression levels were determined using RT-PCR for 35 cycles (A). GAPDH was amplified as loading control (B).

3.11 Expression of 11β-HSD1 and GR mRNA as analysed by qRT-PCR from fresh murine ovarian tumour tissues

RNA was isolated from fresh murine ovarian tumour tissues (irradiated 129S4/SvJae-C57BL/6J loxP-*Stop*-loxP-*K-ras*^{G12D}/*Pten*$^{loxP/loxP}$ mice that had been reconstituted with bone marrow stem cells from lck-Cre GR$^{loxP/loxP}$ or GR$^{loxP/loxP}$ mice) and from healthy ovaries.

cDNA was obtained by reverse transcription and the expression of 11β-HSD1 and GR mRNA were determined by SybrGreen-based real-time quantitative (qRT)-PCR.

A



B

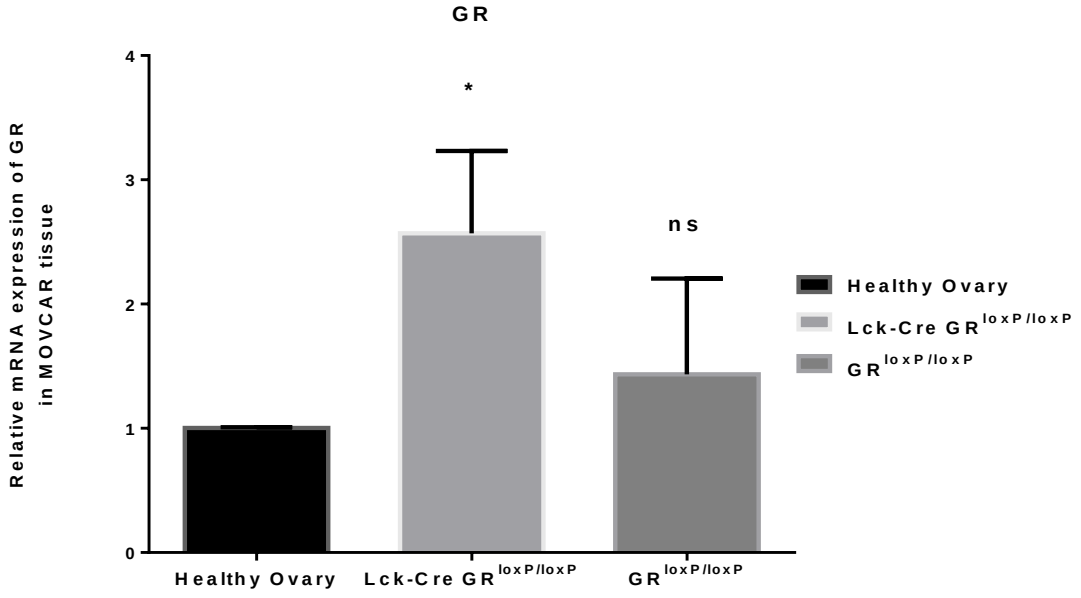
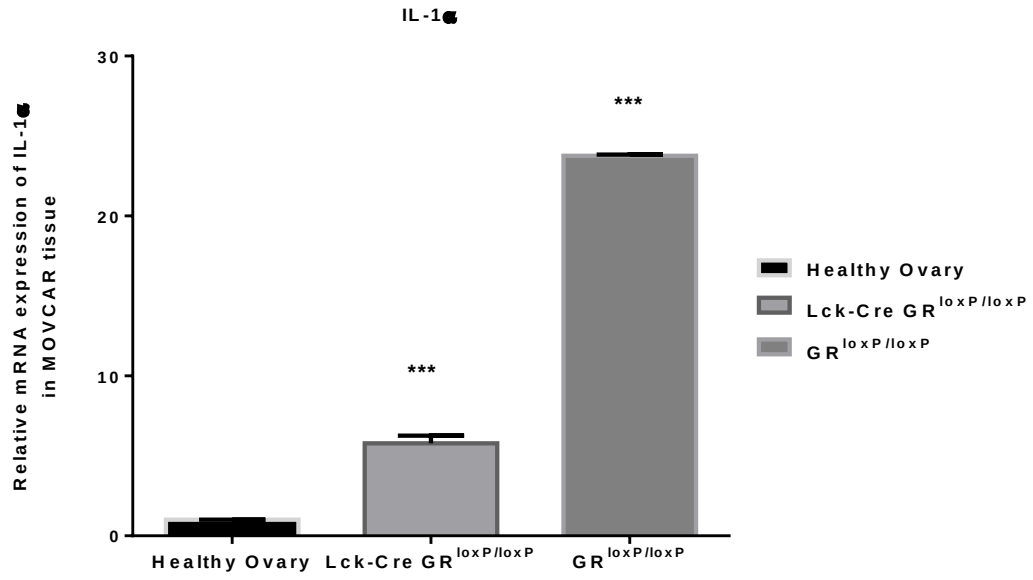


Figure (32): mRNA expression of 11β-HSD1 and GR in murine ovarian cancer tissues. RNA was isolated from murine ovarian cancer tissues and healthy ovaries and reverse transcribed into cDNA. The expression of 11β-HSD1 (n=2) (A) and GR (n=3) (B), mRNA were determined by SybrGreen-based quantitative PCR. The data were analyzed according to $\Delta\Delta C_t$-method. P values were determined by unpaired student t-test using Graphpad Prism 4.

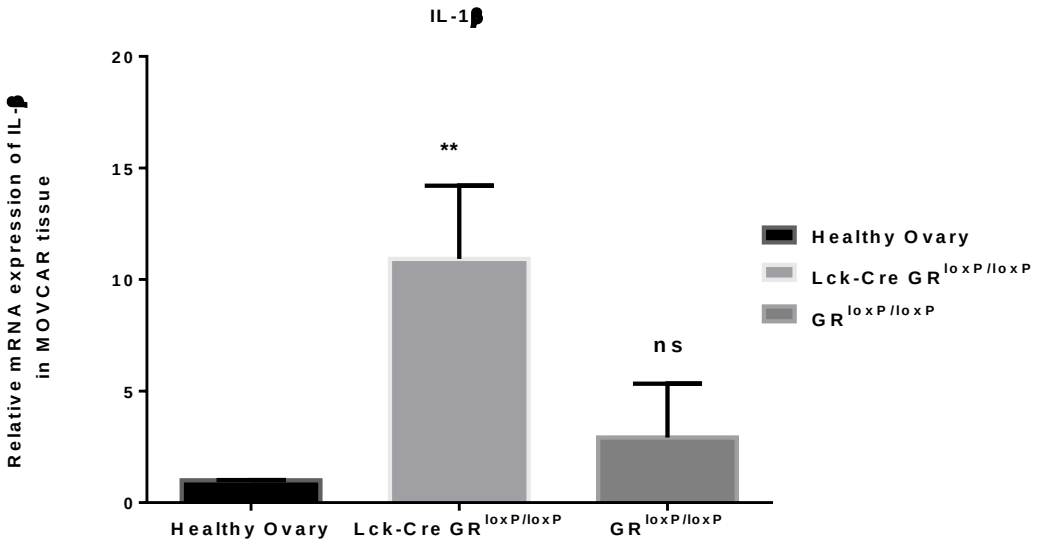
3.12 Expression of IL-1α, IL-1β, TNF-α and MMP-9 mRNA using qRT-PCR in fresh murine ovarian tumour tissues

RNA was isolated from fresh murine ovarian tumour tissue (irradiated 129S4/SvJae-C57BL/6J loxP-*Stop*-loxP-*K-ras*^{G12D}/*Pten*$^{loxP/loxP}$ mice that had been reconstituted with bone marrow stem cells from lck-Cre GR$^{loxP/loxP}$ or GR$^{loxP/loxP}$ mice) and healthy ovaries and reverse-transcribed into cDNA. Then, the relative expression levels of IL-1α, IL-1β, TNF-α and MMP-9 were determined by SybrGreen-based quantitative real-time (qRT)-PCR. This revealed higher levels of IL-1α in the group with GR-responsive T cells (GR$^{loxP/loxP}$) whereas mice harboring GR-deficient Lck Cre GR$^{loxP/loxP}$ T cells displayed higher expression of the inflammatory markers IL-1β and TNF-α and of the TGF-β inducible protease MMP-9. While this is in line with the anti-inflammatory effects mediated via the GR, it cannot be ruled out that GR-deficient T cells were not fully functional either. In this context, a recent publication (Mittelstadt et al., 2012) showed that Lck Cre GR$^{loxP/loxP}$ were immunocompromised due to some defects occurring during positive selection in the thymus.

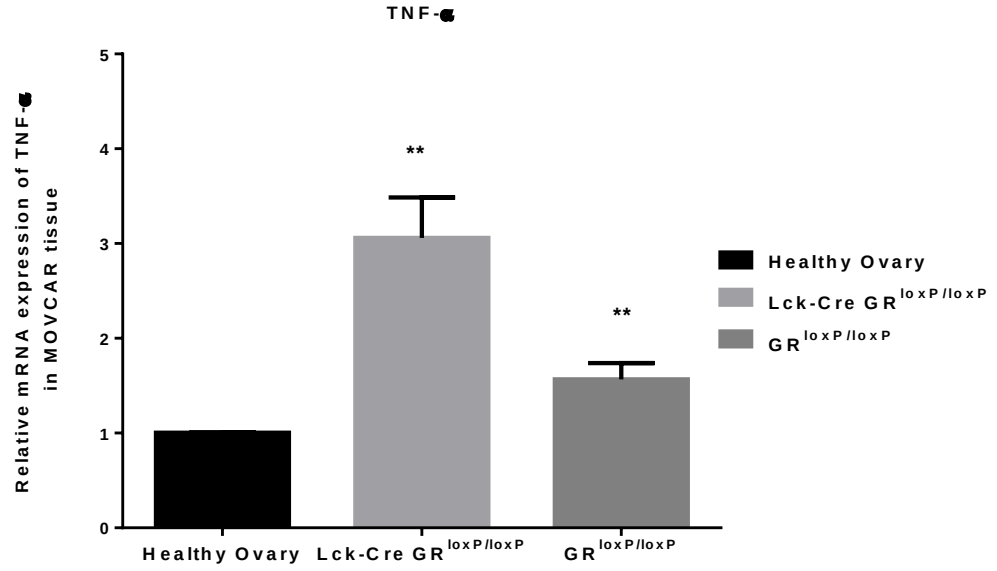
A


IL-1α
Relative mRNA expression of IL-1α in MOVCAR tissue
30
20
10
0
***
***
Healthy Ovary
Lck-Cre GR loxP/loxP
GR loxP/loxP
Healthy Ovary
Lck-Cre GR loxP/loxP
GR loxP/loxP

B


IL-1β
Relative mRNA expression of IL-1β in MOVCAR tissue
20
15
10
5
0
**
ns
Healthy Ovary
Lck-Cre GR loxP/loxP
GR loxP/loxP
Healthy Ovary
Lck-Cre GR loxP/loxP
GR loxP/loxP

C


TNF-α
Relative mRNA expression of TNF-α in MOVCAR tissue
5
4
3
2
1
0
**
**
Healthy Ovary
Lck-Cre GR loxP/loxP
GR loxP/loxP
Healthy Ovary
Lck-Cre GR loxP/loxP
GR loxP/loxP

D

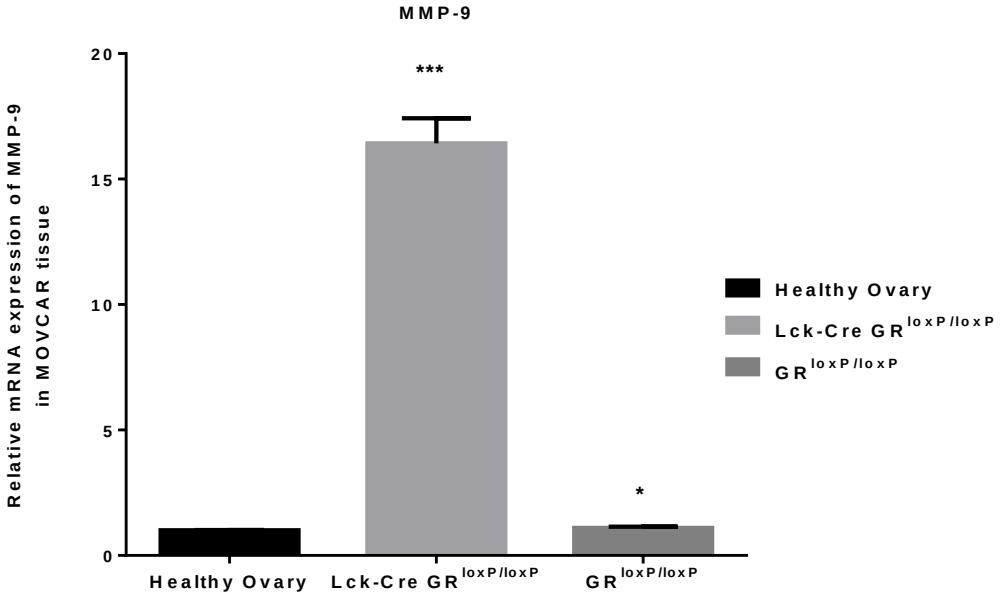

MMP-9
Relative mRNA expression of MMP-9 in MOVCAR tissue
20
15
10
5
0
***
*
Healthy Ovary
Lck-Cre GR loxP/loxP
GR loxP/loxP
Healthy Ovary
Lck-Cre GR loxP/loxP
GR loxP/loxP

Figure (33): mRNA expression of IL-1α, IL-1β, TNF-α and MMP-9 in murine ovarian cancer tissues. RNA was isolated from mouse ovarian cancer tissues and healthy ovaries and reverse-transcribed into cDNA. Expression levels of IL-1α (n=2) (A), IL-1β (n=3) (B), TNF-α (n=3) (C) and MMP-9 (n=2) (D), mRNA were determined by SybrGreen-based quantitative PCR. Data were analyzed according to $\Delta\Delta C_t$-Method. P values were determined by unpaired student t-test using Graphpad Prism 4.

Taken together, these data show that endogenous glucocorticoids play an immunomodulatory role in ovarian cancer. When T cells are rendered resistant against the immunosuppressive effects of glucocorticoids, tumour infiltration by immune cells is significantly improved. However, as further immune-inhibitory mechanisms are at work in the tumour microenvironment, the infiltrating cortisol-resistant T cells cause some inflammation, but mostly assume a regulatory phenotype. Both phenomena may even promote tumour growth (as evidenced by shorter survival or mice that have been adoptively transferred with Lck-Cre $GR^{loxP/loxP}$ cells). Thus, we had to conclude that inhibition of glucocorticoid receptor signaling would not be a promising strategy to improve tumour immune responses. Moreover, two contaminations with mouse parvovirus (MPV) led to a temporary, but long-lasting (18 months) closure of the animal facility which prevented us from performing additional experiments in our mouse model. Instead, we directed our interest towards possible ways of enhancing the functionality of human immune cells (mainly NK cells, in this case) in the presence of glucocorticoids.

3.13 Inhibition of NK cell degranulation by cortisol and TGF-β

First, we confirmed the immunosuppressive effect of cortisol and TGF-β by cell surface staining for the degranulation marker lysosomal-associated membrane protein-1 (LAMP-1) which is also known as CD107a. In cytotoxic immune cells, CD107a is normally found on the vesicular membrane surrounding the cytotoxic granule but not at the cell surface. Upon exocytosis of these granules, however, the vesicle membrane fuses with the cell membrane thereby leading to the sudden appearance of CD107a on the cell surface. Accordingly, degranulation can be assessed via surface staining for CD107a. To investigate the effect of the selected immunosuppressive factors, polyclonal NK cells were treated or not for 72 h with cortisol or TGF-β1 or both. Prior to the staining, the cells were activated with PMA and Ionomycin directly followed by addition of Golgi stop (monensin) to the mixture this stops the exocytosis and the acidification of endosomes. CD107a expression on the cell surface was

then analyzed by extracellular FACS staining. As expected, LAMP-1 was down-regulated by both Cortisol and TGF-β1 which also acted synergistically.

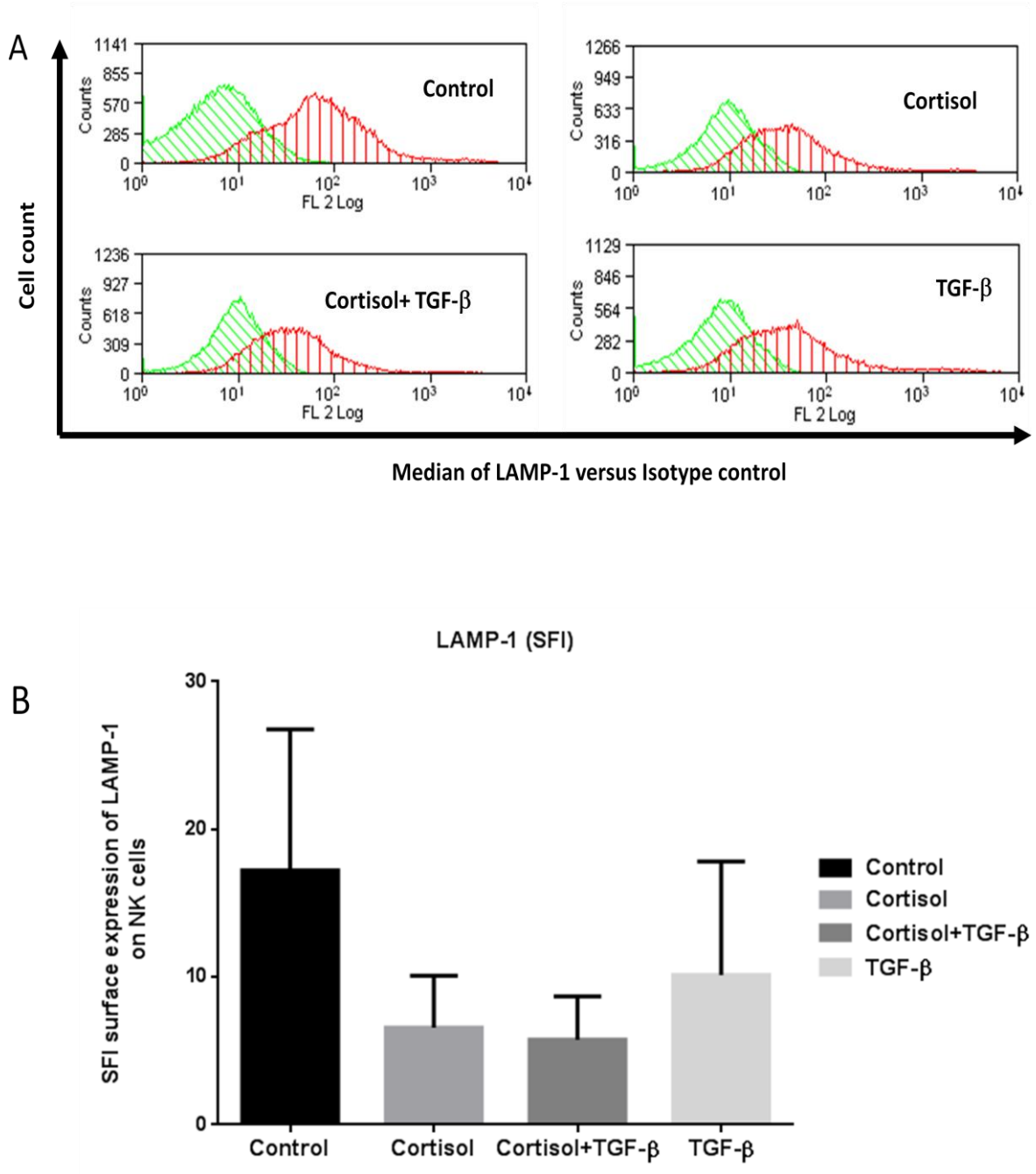


Figure (34): Expression of CD107a on NK cells. Polyclonal NK cells were prepared from PBLs of healthy donor s and either left untreated or treated with 5 µM cortisol or 5ng/ml TGF-β1 or both for 72 h. Then, the cells were stimulated with PMA/Ionomycin followed by Golgi stop (1:1000, 6µg/ml) for 4 h. Surface expression of CD107a on $CD3^{-}CD56^{+}$, i.e. NK cells, was analyzed by flow cytometry (A). To obtain specific fluorescence intensity (SFI) values, the mean fluorescence values obtained with a PE-conjugated anti-CD107a antibody

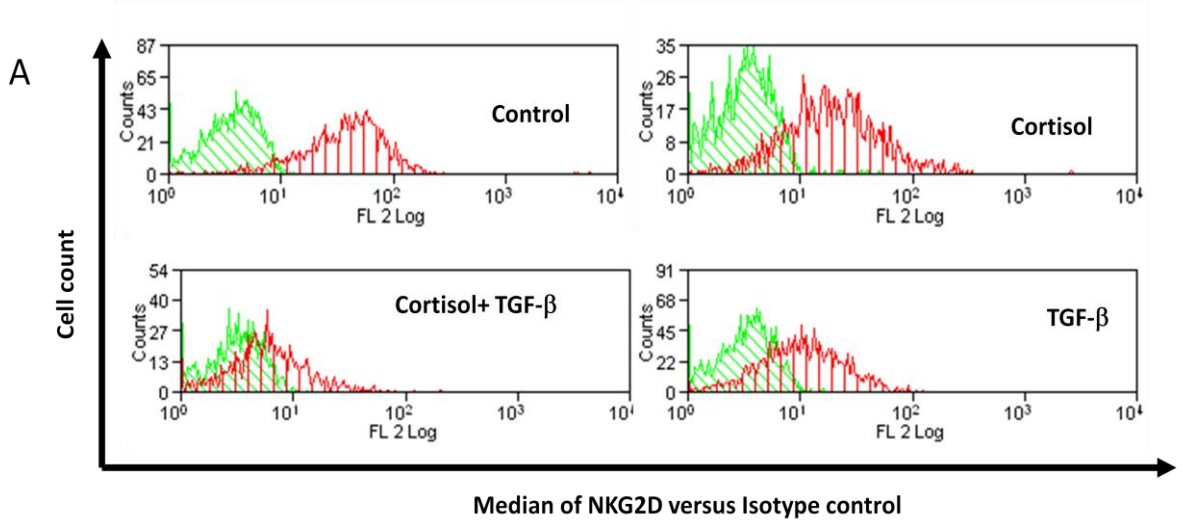
were divided by the mean fluorescence values obtained with a PE-labeled isotype control antibody (B). A representative experiment (n=2) is shown.

3.14 Activating receptors on NK cells and their response to cortisol and TGF-β

In order to understand how NK cells might still be activated in the presence of endogenous glucocorticoids, we assessed the effects of cortisol on activating NK cell receptors. Since TGF-β (which is abundant in the tumour microenvironment) is known to suppress expression of several of these receptors, TGF-β1was included as control.

3.14.1 Expression of NKG2D on NK cells

NKG2D is an activating receptor expressed on NK cells, CD8 T cells and further cell types. Primary polyclonal NK cells were treated with cortisol or TGF-β1 or both (cortisol /TGF-β1) or left untreated as control. Expression of NKG2D on NK cells was analyzed by flow cytometry. The result indicated that NKG2D is downregulated by both cortisol and TGF-β1. Cortisol and TGF-β1 were further found to act synergistically in downregulating NKG2D.



B

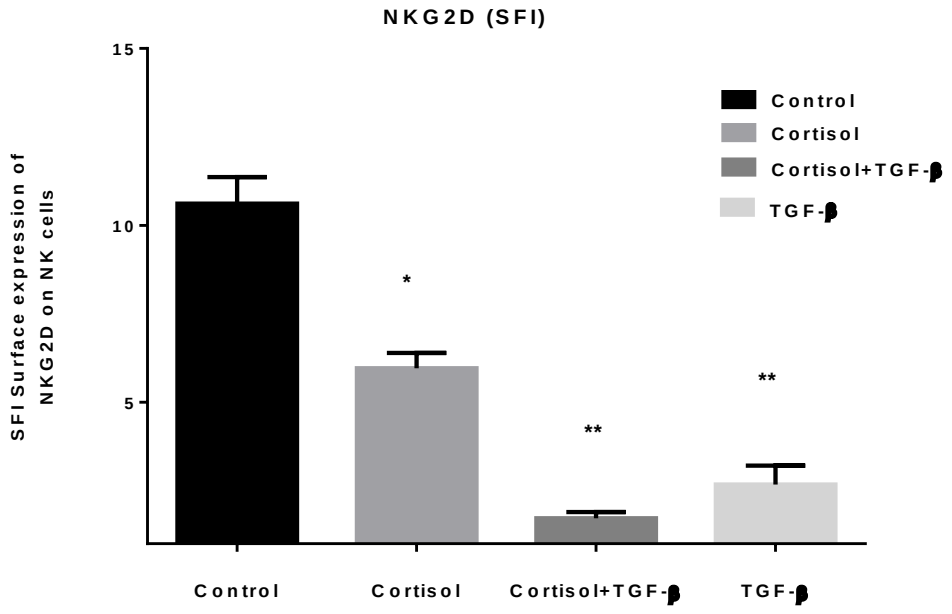


Figure (35): Expression of NKG2D on NK cells. Polyclonal NK cells were prepared from PBLs of healthy donors and either left untreated or treated with 5 µM cortisol or 5ng/ml TGF-β1 or both for 72 h. NKG2D surface expression was analyzed on $CD3^{-}CD56^{+}$ cells (i.e. NK cells) by flow cytometry (A). To obtain specific fluorescence intensity (SFI) values, the mean fluorescence values obtained with a PE-conjugated anti-NKG2D antibody were divided by the mean fluorescence values obtained with a PE-labeled isotype control antibody (B). A representative experiment (n=5) is shown. P values were determined by unpaired student t-test using Graphpad Prism 4.

To explore potential effects of cortisol and TGF-β1 on NKG2D mRNA levels, polyclonal NK cells were treated for 6 h with cortisol or TGF-β1 or both or left untreated as control. RNA was isolated, reverse-transcribed and NKG2D mRNA expression was quantified by qRT-PCR. Similar to the surface expression data this revealed a synergistic down-regulation of NKG2D by both cortisol and TGF-β1.

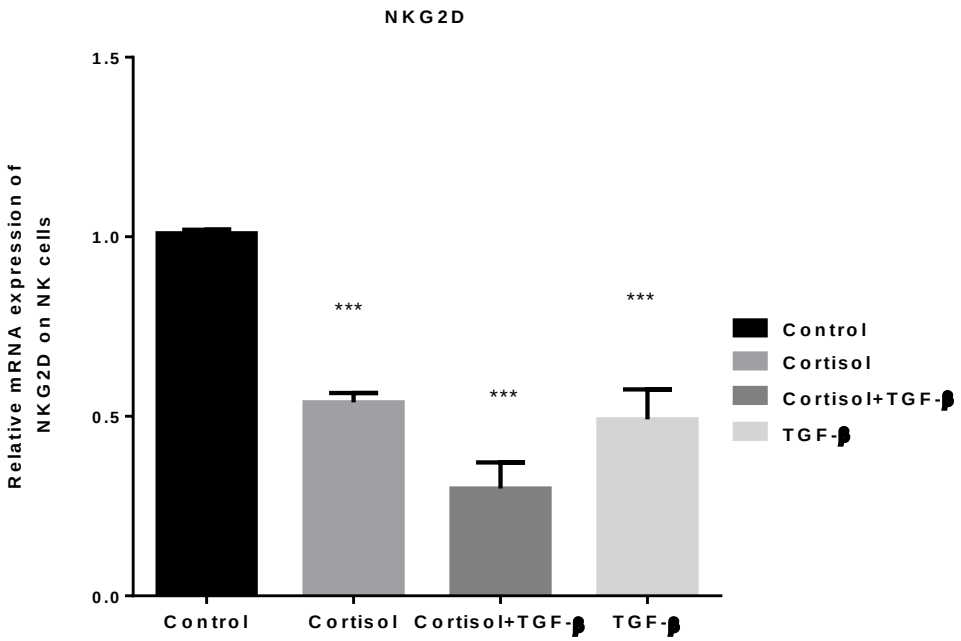


Figure (36): NKG2D mRNA expression in NK cells. RNA was isolated from NK cells that had been left untreated or been treated for 6 h with 5 µM cortisol or 5 ng/ml TGF-β1 or both. After reverse transcription into cDNA, relative expression of NKG2D was analyzed by SybrGreen-based quantitative PCR using the following primers: 5´-TCTCGACACAGCTGGGAGATG-3´ and 5´-GACATCTTTGCTTTTGCCATCGTG-3´ .18S rRNA was used as internal control. Data were analyzed according to $\Delta\Delta C_t$-method (n=3). P values were determined by unpaired student t-test using Graphpad Prism 4.

3.14.2 Expression of natural cytotoxicity receptors on NK cells

Natural cytotoxicity receptors (NCRs) include NKp46, NKp44 and NKp30. Polyclonal NK cells were again treated for 72 h with cortisol or TGF-β1 or both or left untreated as control. Expression levels for the different NCRs on NK cells were analyzed by flow cytometry. Surprisingly, this revealed that NKp46 is even up-regulated in response to cortisol, but down-regulated with TGF-β1, always using the untreated control for comparison.

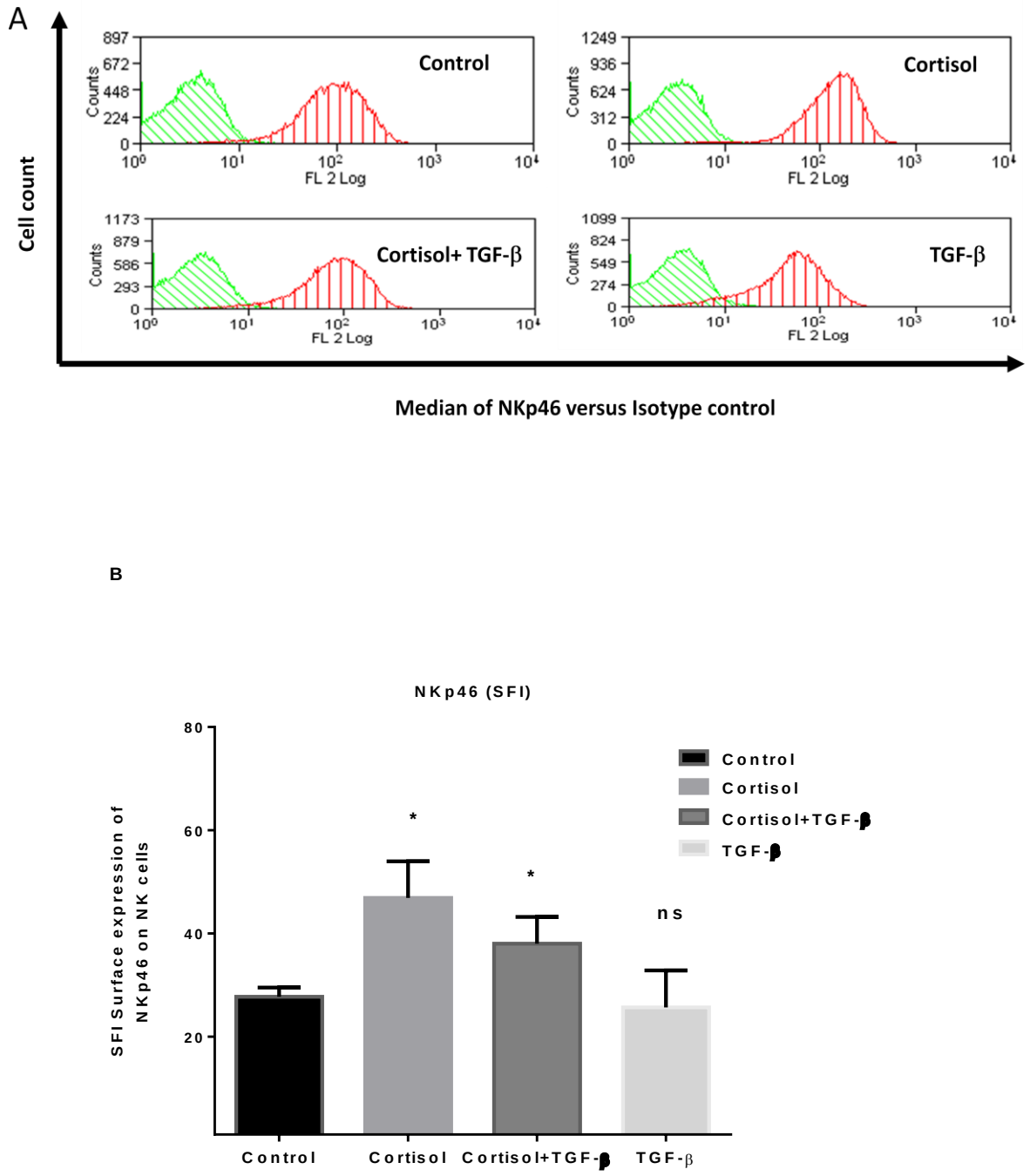


Figure (37): Expression of NKp46 on NK cells. Polyclonal NK cells were prepared from PBLs of healthy donors and either left untreated or treated with 5 µM cortisol or 5ng/ml TGF-β1 or both for 72 h. The surface expression of NKp46 was analyzed on $CD3^{-}CD56^{+}$ NK cells (A) and specific fluorescence intensities (SFI values) were calculated for NKp46 (B). A representative experiment (n=4) is shown. P values were determined by unpaired student t-test using Graphpad Prism 4.

mRNA data, in contrast, showed a slight downregulation of NKp46 mRNA with cortisol and a much more pronounced reduction in NKp46 mRNA levels in response to TGF-β1. Still, the mRNA data support the finding that NKp46 expression remains largely intact after treatment with cortisol.

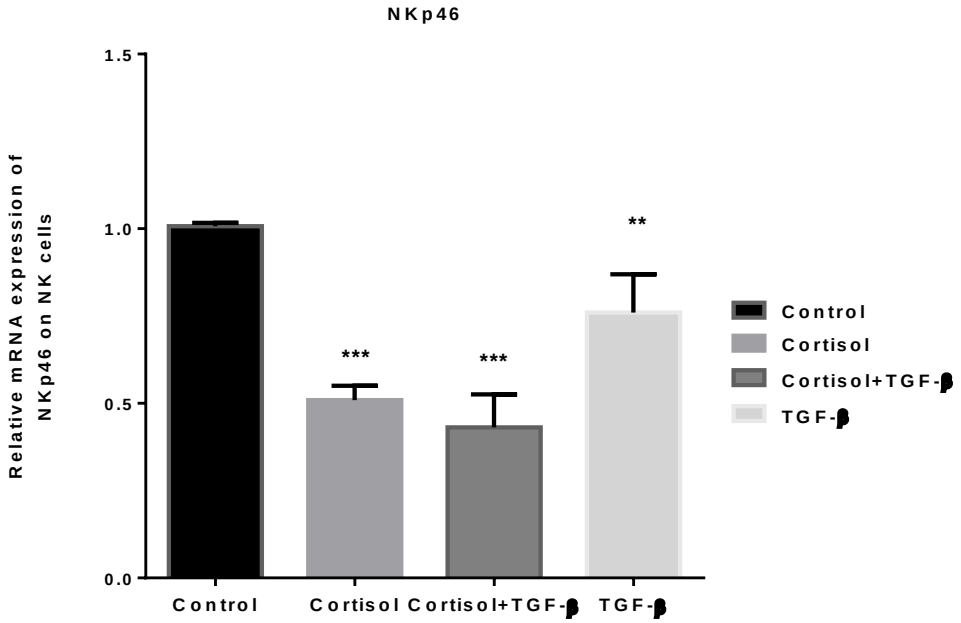


Figure (38): NKp46 mRNA expression in NK cells. RNA was isolated from NK cells that had been treated for 6 h with 5 µM cortisol or 5ng/ml TGF-β1 or both. After reverse transcription into cDNA, expression of NKp46 was analyzed relative to an untreated control, using SybrGreen-based quantitative PCR with the following primers: 5´-ACAACCATGCCTGGTCTTTC-3´and 5´-AAAAGGTAGCGTGCCCCAAGT-3´.18S rRNA was used as internal control. Data were analyzed according to $\Delta\Delta C_t$-method (n=3). P values were determined by unpaired student t-test using Graphpad Prism 4.

For NKp44, the result showed that NKp44 is down-regulated in response to cortisol treatment whereas TGF-β1 has no effect on NKp44.

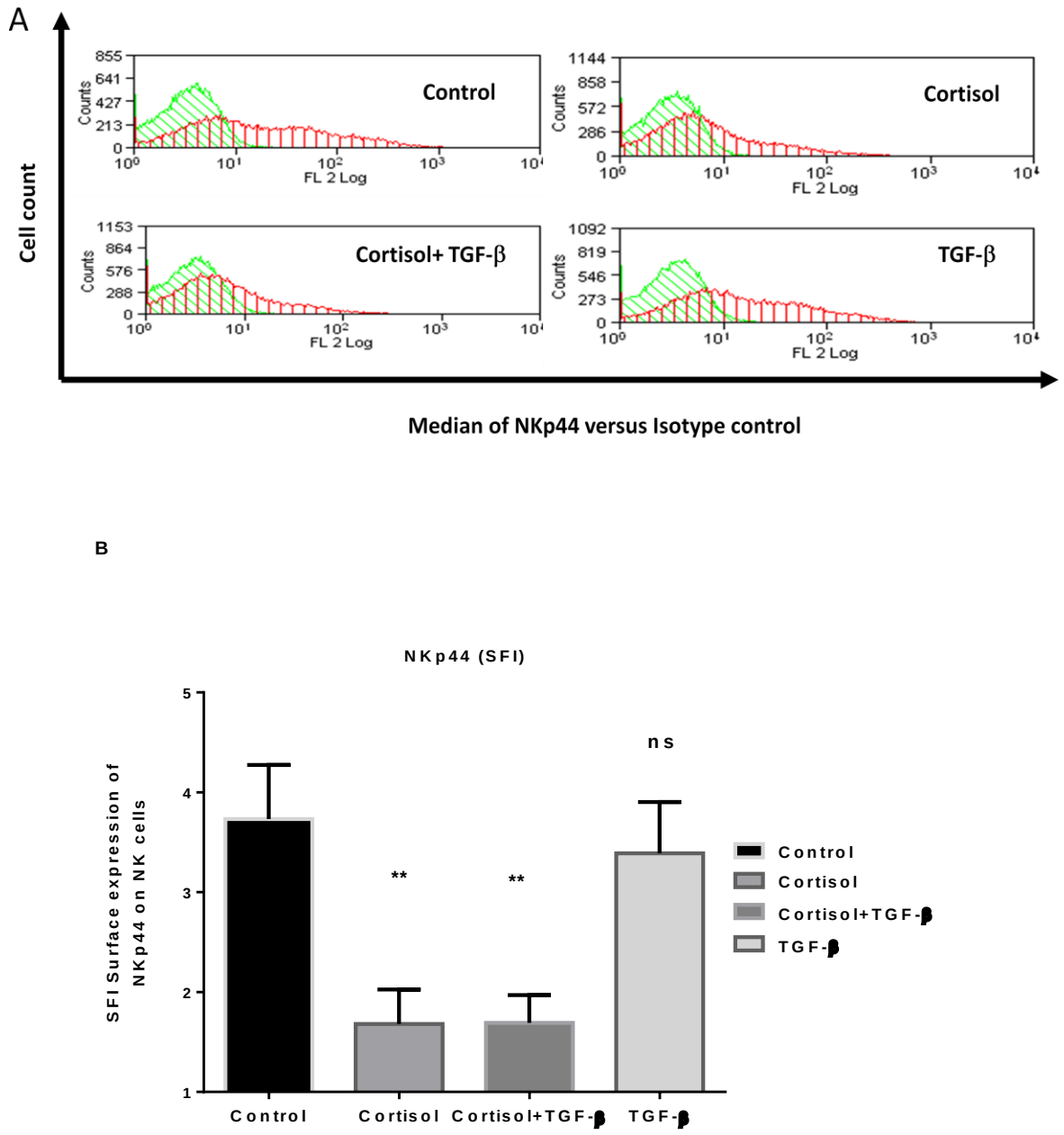


Figure (39): Expression of NKp44 on NK cells. Polyclonal NK cells were prepared from PBLs of healthy donors and either left untreated or treated with 5 µM cortisol or 5 ng/ml TGF-β1 or both for 72 h. Surface expression of NKp44 on $CD3^{-}CD56^{+}$ NK cells was analyzed by flow cytometry (A) and specific fluorescence intensities (SFI values) were calculated (B). A representative experiment (n=3) is shown. P values were determined by unpaired student t-test using Graphpad Prism 4.

On mRNA level, NKp44 expression was not detected in cortisol or TGF-β1 treated NK cells, but was detected in the control.

NKp30, in contrast, was down-regulated by either cortisol or TGF-β1 with both shown a synergistic effect on protein and mRNA level.

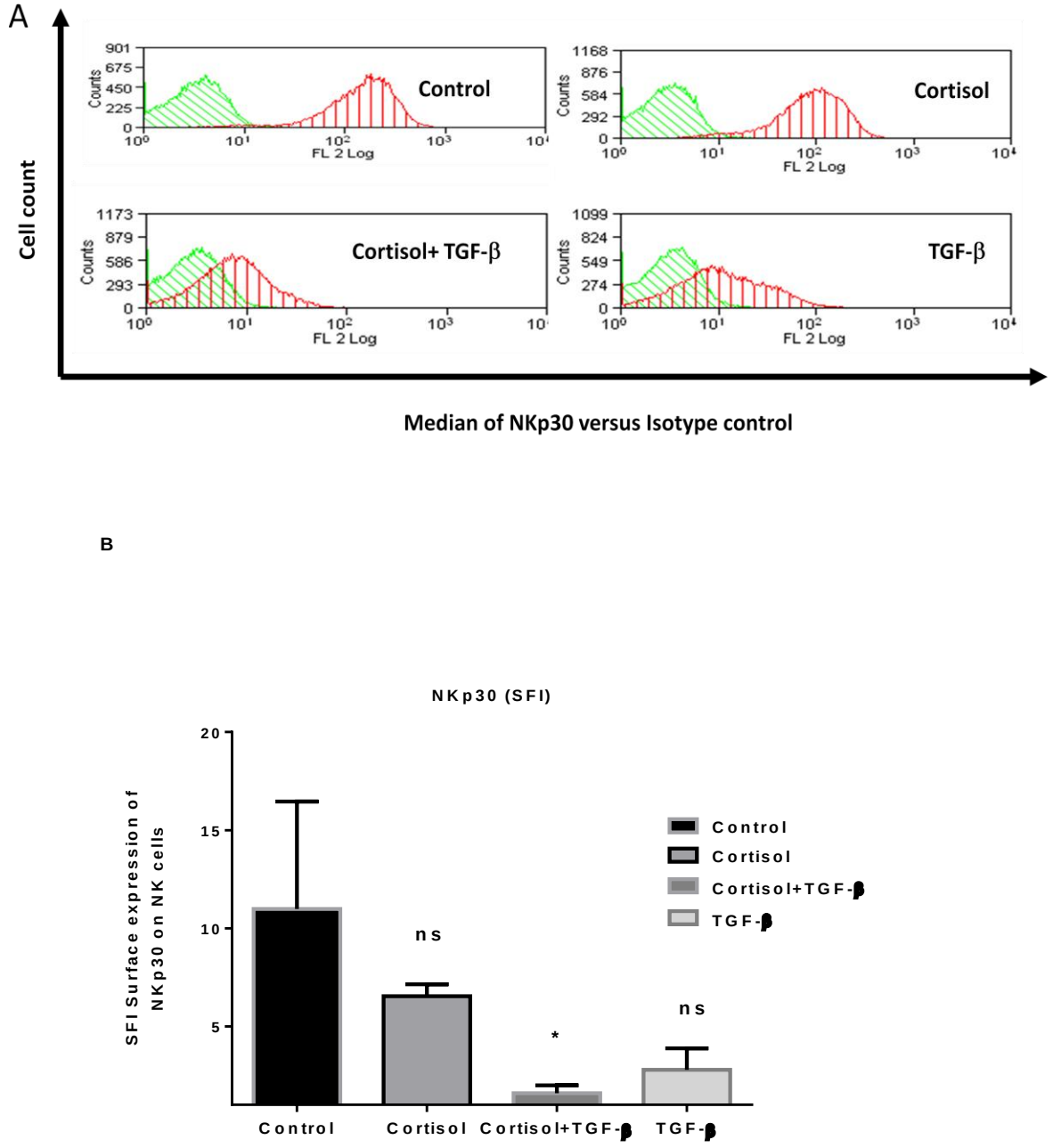


Figure (40): Expression of NKp30 on NK cells. Polyclonal NK cells were prepared from PBLs of healthy donors and either left untreated or treated with 5 µM cortisol or 5 ng/ml TGF-β1 or both for 72 h. Surface expression of NKp30 on $CD3^{-}CD56^{+}$ NK cells was analyzed by flow cytometry (A) and SFI values were determined (B). A representative experiment (n=3) is shown. P values were determined by unpaired student t-test using Graphpad Prism 4.

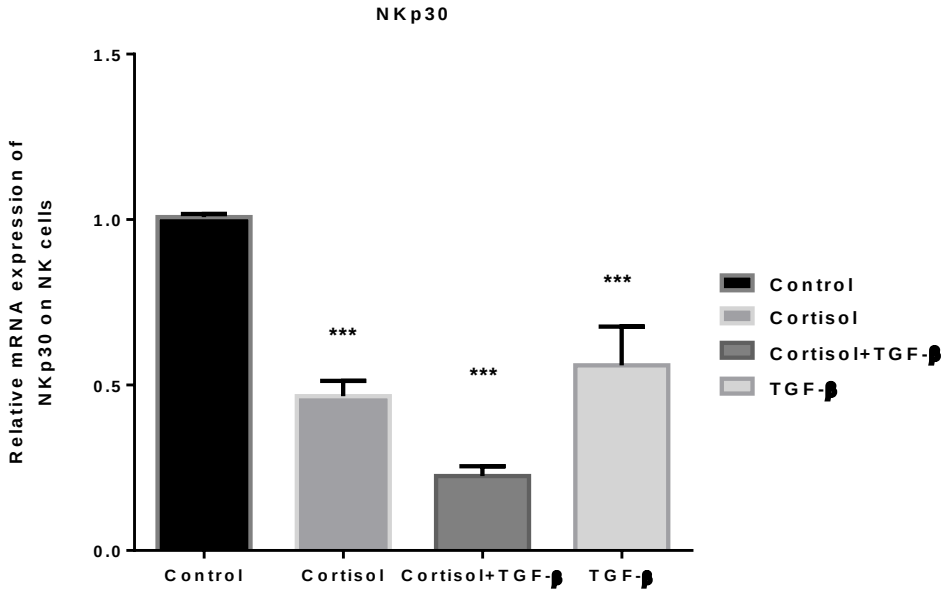


Figure (41): NKp30 mRNA expression in NK cells. RNA was isolated from NK cells that had been treated for 6 h with 5 µM cortisol or 5ng/ml TGF-β1 or both. After reverse transcription into cDNA, expression of NKp30 was analyzed relative to an untreated control, using SybrGreen-based quantitative PCR with the following primers: 5´-TGTCCTGAGAAATGGGAAGG-3´and 5´-ACAGTGTTCAGGGACCCAAG-3´ .18S rRNA was used as internal control. Data were analyzed according to $\Delta\Delta C_t$-method (n=3). P values were determined by unpaired student t-test using Graphpad Prism 4.

3.14.3 Expression of DNAM-1 on cortisol- or TGF-β-treated NK cells

DNAX accessory molecule-1 (DNAM-1), also known as CD226, is an activating NK cell receptor that was found to mediate a major part of NK cell cytotoxicity against cancer cells including cell lines derived from ovarian carcinoma (Carlsten et al., 2009). Polyclonal NK cells were thus treated for 72 h with cortisol or TGF-β1 or both or left untreated as control. The expression of DNAM-1 on NK cells was analyzed by flow cytometry. The result indicated that cortisol does not affect DNAM-1 surface expression whereas TGF-β1 is able to (slightly) downregulate DNAM-1 on NK cells.

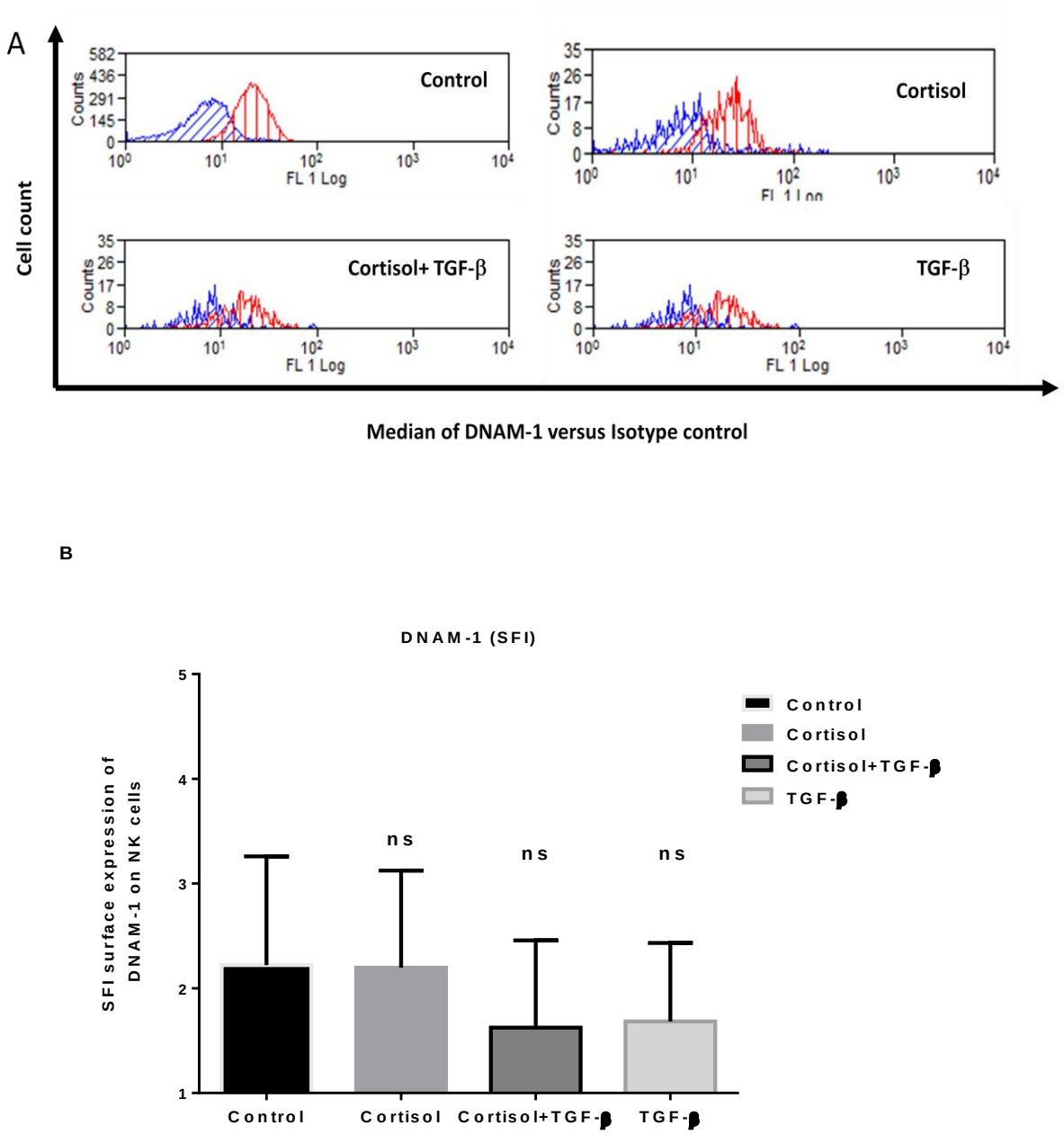


Figure (42): Expression of DNAM-1 on NK cells. Polyclonal NK cells were prepared from PBLs of healthy donors and either left untreated or treated with 5 µM cortisol or 5 ng/ml TGF-β1 or both for 72 h. Surface expression of DNAM-1 (CD226) on $CD3^{-}CD56^{+}$ NK cells was analyzed by flow cytometry (A) and SFI values were determined (B). A representative experiment (n=3) is shown. P values were determined by unpaired student t-test using Graphpad Prism 4.

On mRNA level, DNAM-1 expression was slightly up-regulated by either cortisol or TGF-β.

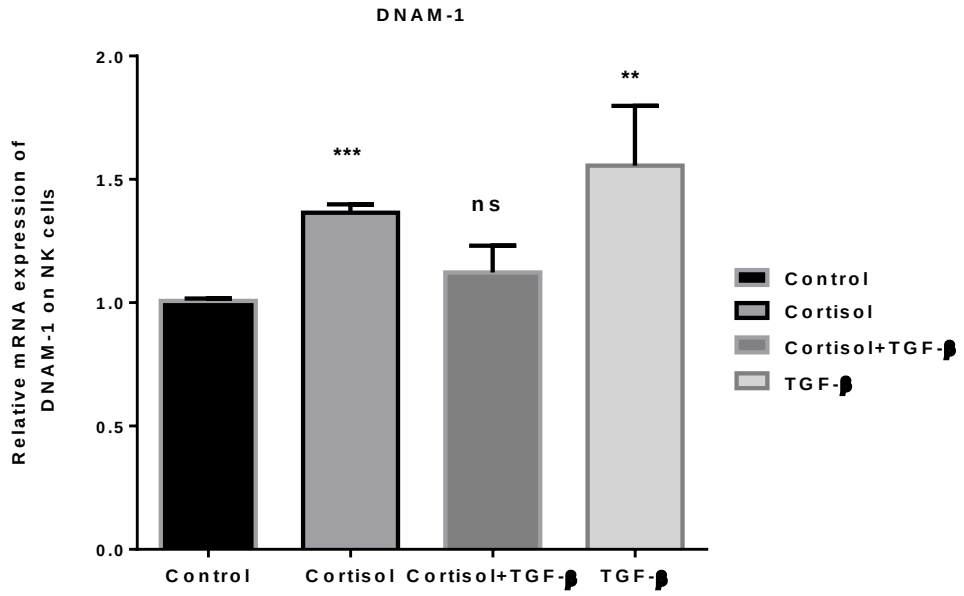


Figure (43): DNAM-1 mRNA expression in NK cells. RNA was isolated from NK cells that had been treated for 6 h with 5 µM cortisol or 5ng/ml TGF-β1 or both. After reverse transcription into cDNA, expression of DNAM-1 mRNA was analyzed relative to an untreated control, using SybrGreen-based quantitative PCR with the following primers: 5´-GCAGTGACCAAGAGGGTGTT-3´ and 5´-CTGCTCGCTCTAACGCTTCT-3´.18S rRNA was used as internal control. Data were analyzed according to $\Delta\Delta C_t$-method (n=3). P values were determined by unpaired student t-test using Graphpad Prism 4.

3.14.4 Expression of 2B4 on NK cells

2B4 (CD244) is an activating NK cell receptor which mainly functions as co-receptor rather than as true receptor. To explore a potential regulation of 2B4 by cortisol or TGF-β1, polyclonal NK cells were again treated for 72 h with cortisol or TGF-β1 or both or left untreated as control. The expression of 2B4 on NK cells was analyzed by flow cytometry. The result showed that 2B4 expression remains basically unaltered in the presence of cortisol or TGF-β1.

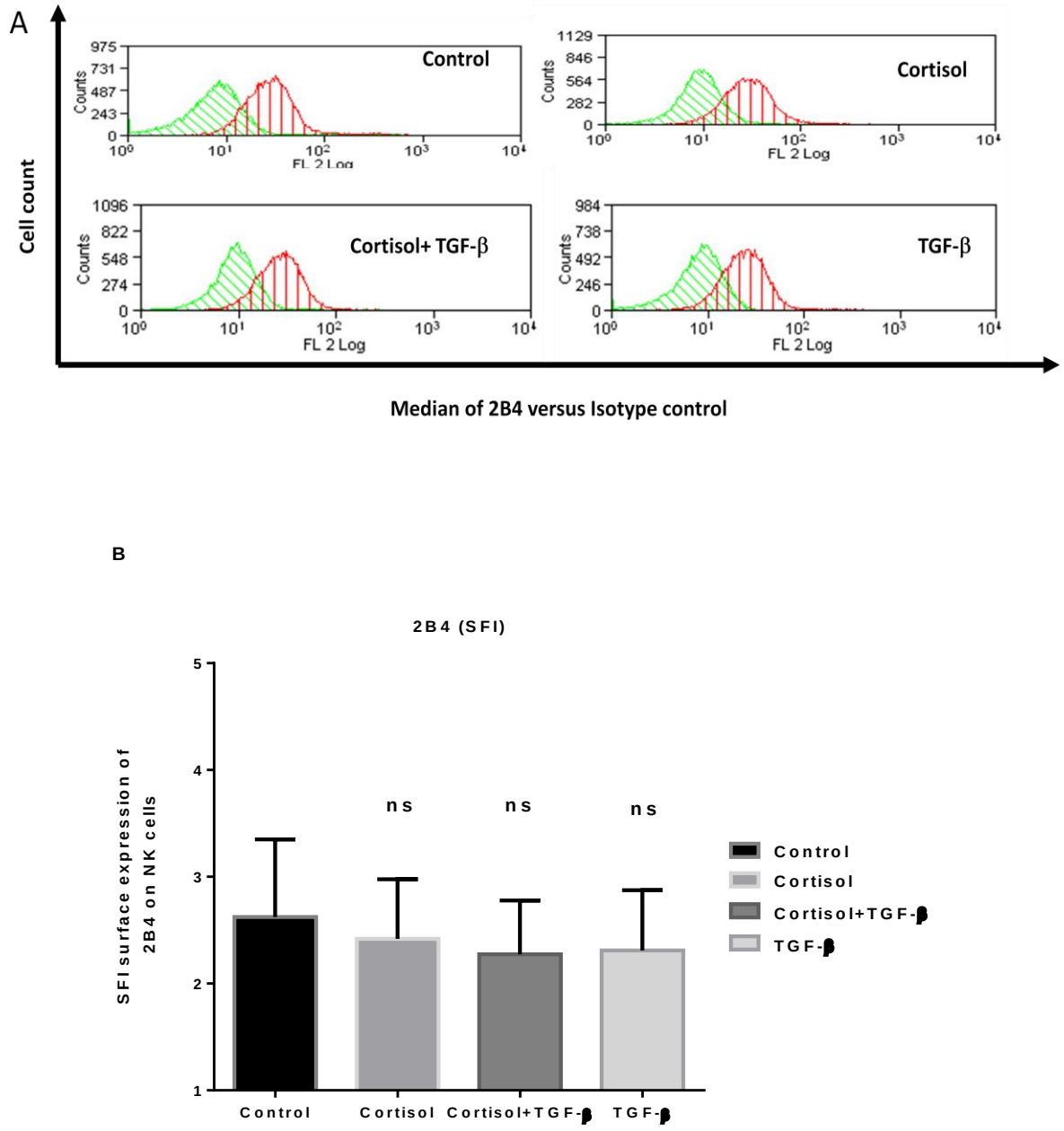


Figure (44): Expression of 2B4 on NK cells. Polyclonal NK cells were prepared from PBLs of healthy donors and either left untreated or treated with 5 µM cortisol or 5 ng/ml TGF-β1 or both for 72 h. Surface expression of 2B4 (CD244) on $CD3^{-}CD56^{+}$ NK cells was analyzed by flow cytometry (A) and SFI values were determined (B). A representative experiment (n=3) is shown. P values were determined by unpaired student t-test using Graphpad Prism 4.

On mRNA level, however, 2B4 was hardly affected by cortisol and slightly up-regulated by TGF-β.

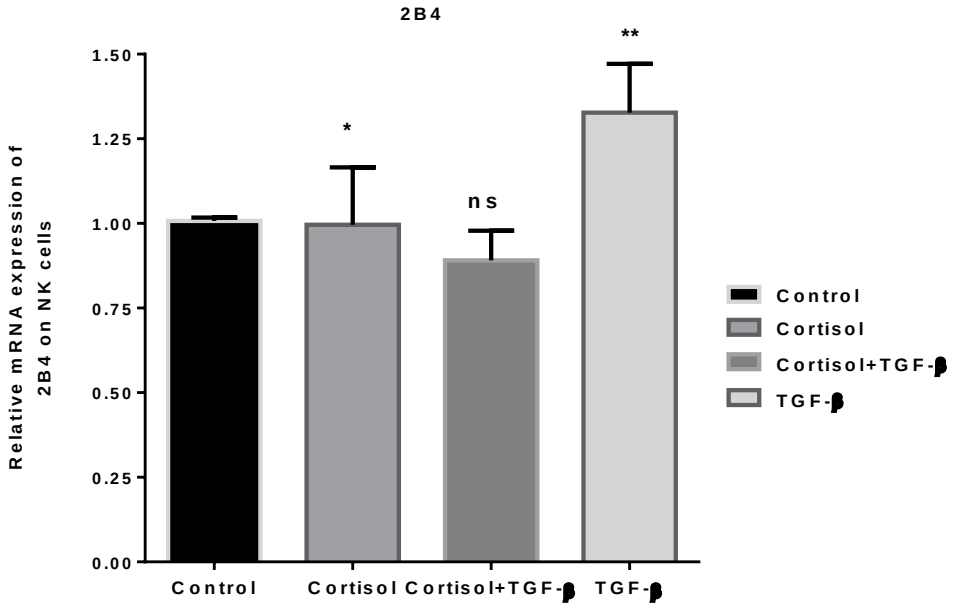


Figure (45): 2B4 (CD244) mRNA expression in NK cells. RNA was isolated from NK cells that had been treated for 6 h with 5 µM cortisol or 5ng/ml TGF-β1 or both. After reverse transcription into cDNA, expression of 2B4 mRNA was analyzed relative to an untreated control, using SybrGreen-based quantitative PCR with the following primers: 5´-ACAAAAGGCCACATTCCAAG-3´ and 5´-AACCCAGAGAGGGGAGAAAA-3´ .18S rRNA was used as internal control. Data were analyzed according to $\Delta\Delta C_t$-method (n=3). P values were determined by unpaired student t-test using Graphpad Prism 4.

3.14.5 Expression of CD2 on NK cells

The last factor investigated on cortisol- or TGF-β1 -treated NK cells was the co-stimulatory molecule. Polyclonal NK cells were again treated for 72 h with cortisol or TGF-β1 or both or left untreated as control. Expression of CD2 on NK cells was analyzed by flow cytometry. The result indicated that CD2 is down-regulated with Cortisol or TGF-β1.

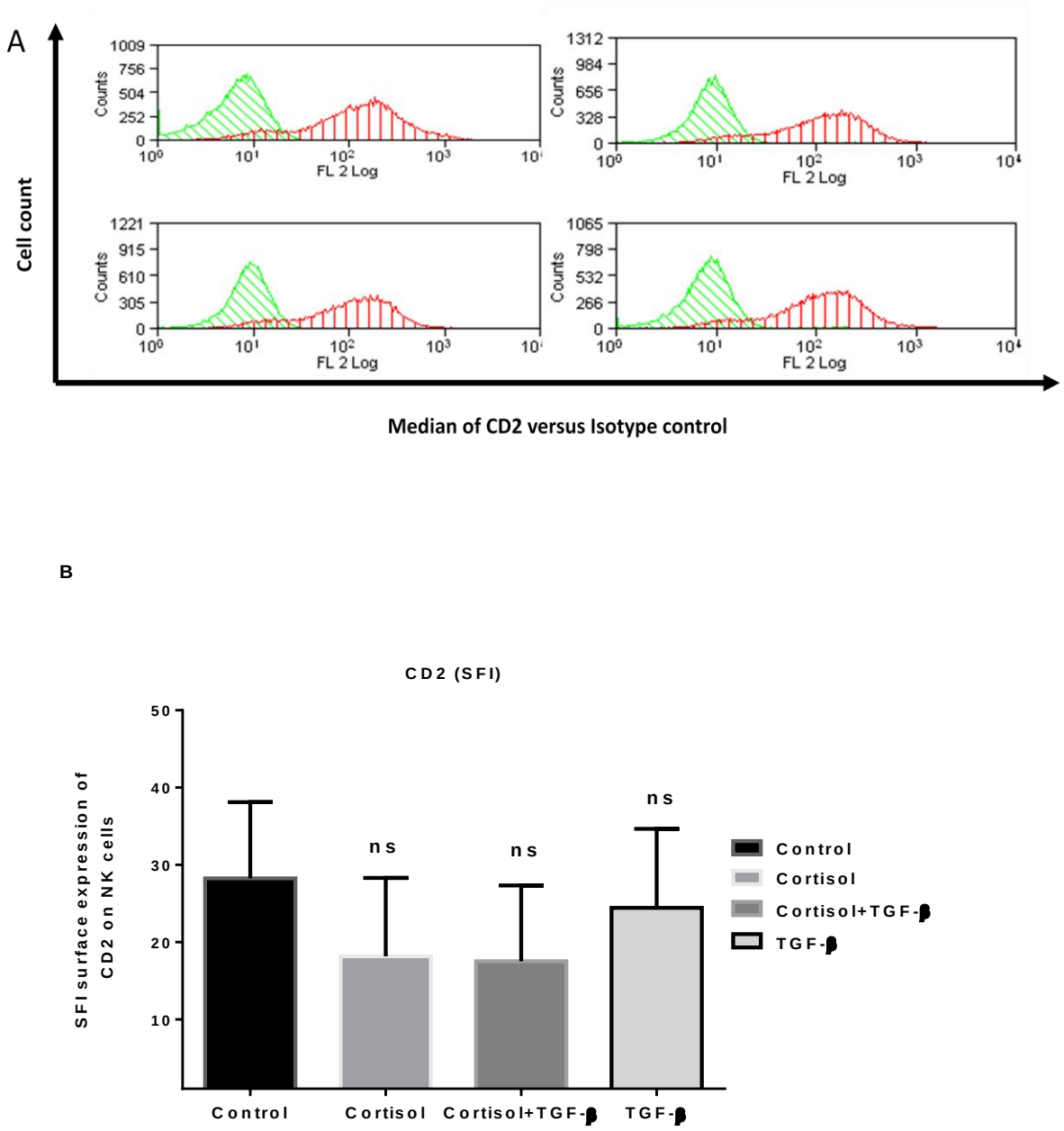


Figure (46): Expression of CD2 on NK cells. Polyclonal NK cells were prepared from PBLs of healthy donors and either left untreated or treated with 5 µM cortisol or 5 ng/ml TGF-β1 or both for 72 h. Surface expression of CD2 on $CD3^{-}CD56^{+}$ NK cells was analyzed by flow cytometry (A) and SFI values were determined (B). A representative experiment (n=3) is shown. P values were determined by unpaired student t-test using Graphpad Prism 4.

On mRNA level, downregulation of CD2 could be observed with Cortisol and hardly affected by TGF-β, with both factors acting in synergy.

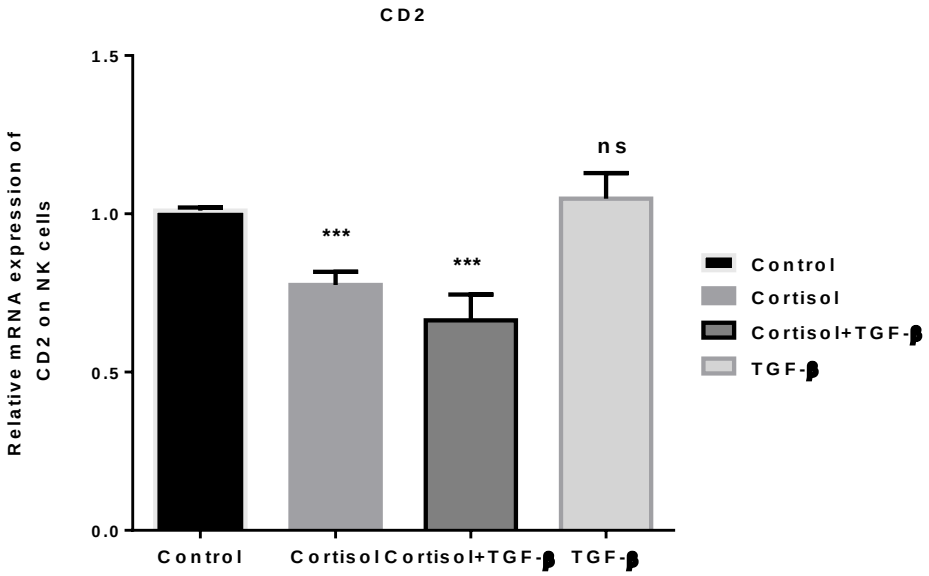


Figure (47): CD2 mRNA expression in NK cells. RNA was isolated from NK cells that had been treated for 6 h with 5 µM cortisol or 5ng/ml TGF-β1 or both. After reverse transcription into cDNA, expression of 2B4 mRNA was analyzed relative to an untreated control, using SybrGreen-based quantitative PCR with the following primers: 5´-CCAGCCTGAGTGCAAAATTCA-3´and 5´-GACAGGCTCGACACTGGATTC-3´ .18S rRNA was used as internal control. Data were analyzed according to $\Delta\Delta C_t$-method (n=3). P values were determined by unpaired student t-test using Graphpad Prism 4.

A brief summary showing how the different NK cell receptors are affected by cortisol and/or β is given in the table below.

	downregulation at the cell surface			downregulation on mRNA level			residual surface expression		
	cortisol	**TGF-β1**	**synergy**	**cortisol**	**TGF-β1**	**synergy**	**cortisol**	**TGF-β1**	**synergy**
NKG2D	++	+++	+	+	+	+	+	(+)	-
NKp30	+	++	++	++	++	+	++	+	(+)
NKp44	++	+	-				+	+++	+
NKp46	-	-	-	+	+	+	+++	++	+++
CD226	-	+	+	-	-	-	+++	++	++
2B4	-	-	-	-	-	-	+++	+++	+++
CD2	-	-	-	+	-	+	++	++	++

Table (12): Summary on the observed effects of cortisol and/or TGF-β1 on different activating NK cell receptors. Synergy was rated as positive when there was at least an additive effect.

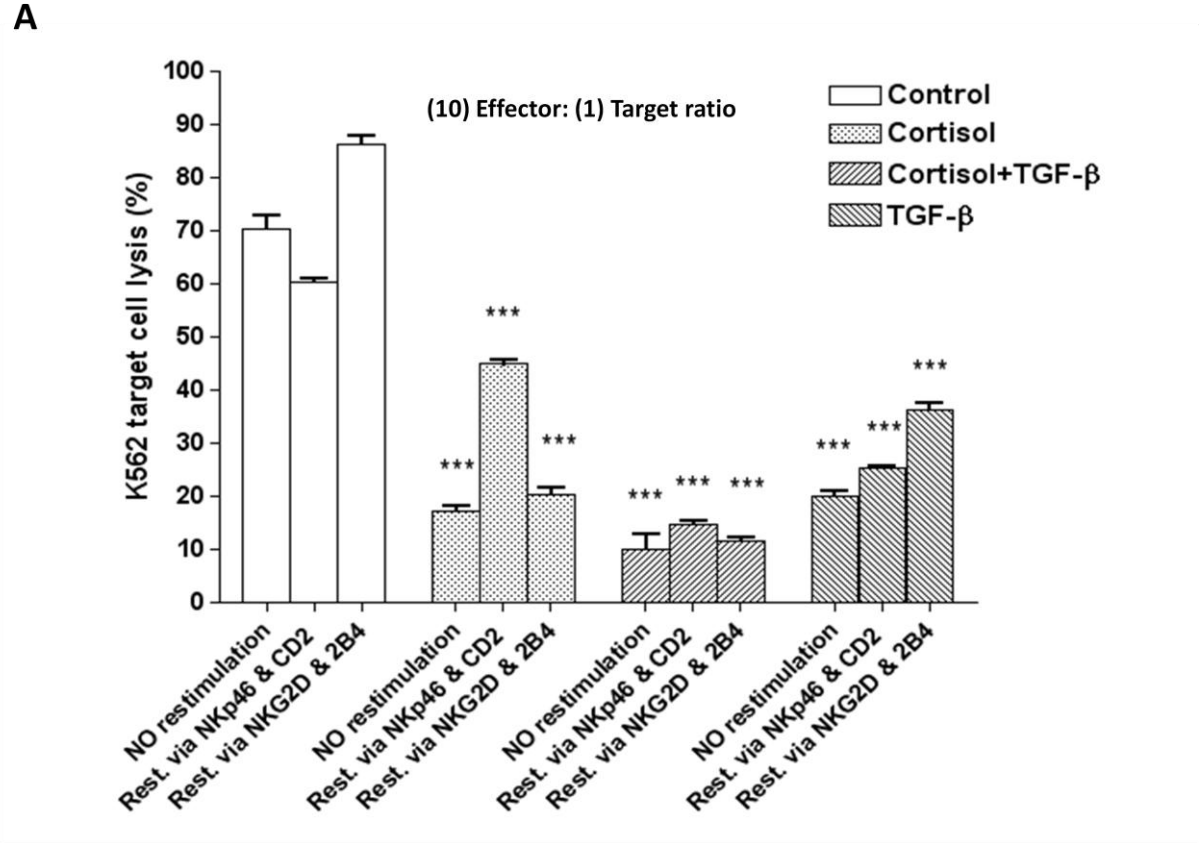
3.15 Restimulation experiment on NK cells that had been treated with cortisol or TGF-β1

As shown in the first part of this thesis, cortisol levels are greatly elevated in ovarian cancer. This is most likely due to an increased expression of the enzyme 11β-HSD1 on myeloid cells. 11β-HSD1 is known to regulate local glucocorticoid levels by conversion of biologically inactive cortisone into active cortisol. In a mouse model of ovarian cancer, however, adoptive transfer of glucocorticoid-insensitive T cells from Lck-Cre $GR^{loxP/loxP}$ mice improved intratumoral T cell infiltration but worsened the outcome which may be related to an induced T_{reg} phenotype that was observed in most T cells in the tumour microenvironment. Accordingly, we tried to devise a strategy for activating natural killer cells in the presence of immunosuppressants like cortisol or TGF-β1. In the ideal case, NK cell activation could modulate the tumour microenvironment and make it more favorable for subsequent T cell responses.

In this context, we found that the activating NK cell receptor NKp46 was not only resistant towards cortisol or TGF-β1; it even displayed an enhanced surface expression upon treatment of NK cells with cortisol. This raises the question whether NKp46 signalling could still be activated in NK cells even in the presence of glucocorticoids and whether this could restore the killing capacity of NK cells against tumour cells. This was tested by a restimulation experiment during which cortisol- or TGF-β1-treated NK cells were activated either with agonistic NKp46 and CD2 antibodies (that were coupled to magnetic beads). As control for a cortisol- and TGF-β1-sensitive pathway, plate-bound antibodies against NKG2D and 2B4 were used. This revealed that beads loaded with NKp46 and CD2 antibodies could largely restore the killing capacity of cortisol-treated NK cells against K562 tumour cell targets. In contrast, no rescue was achieved in TGF-β1 treated NK cells. In line with the strong down-regulation of NKG2D in response to either cortisol or TGF-β1, restimulation with plate-bound antibodies against NKG2D and 2B4 was altogether less effective even though a slight increase in killing capacity was achieved here for TGF-β1 treated cells. NK cells treated with both TGF-β1 and cortisol were refractory to either stimulation. Thus, we conclude that NK cells can still be activated in the presence of immunosuppressive cytokines. However, restimulation has to occur via a pathway that is not fully incapacitated by the respective immunosuppressive factors and is thus highly context-dependent. This may become extremely

difficult when several immune-inhibitory factors are simultaneously present (which, unfortunately, is the case in the tumour microenvironment). Accordingly, relief of immunosuppression may become an important addition to current immunotherapeutic regimens.

A



B

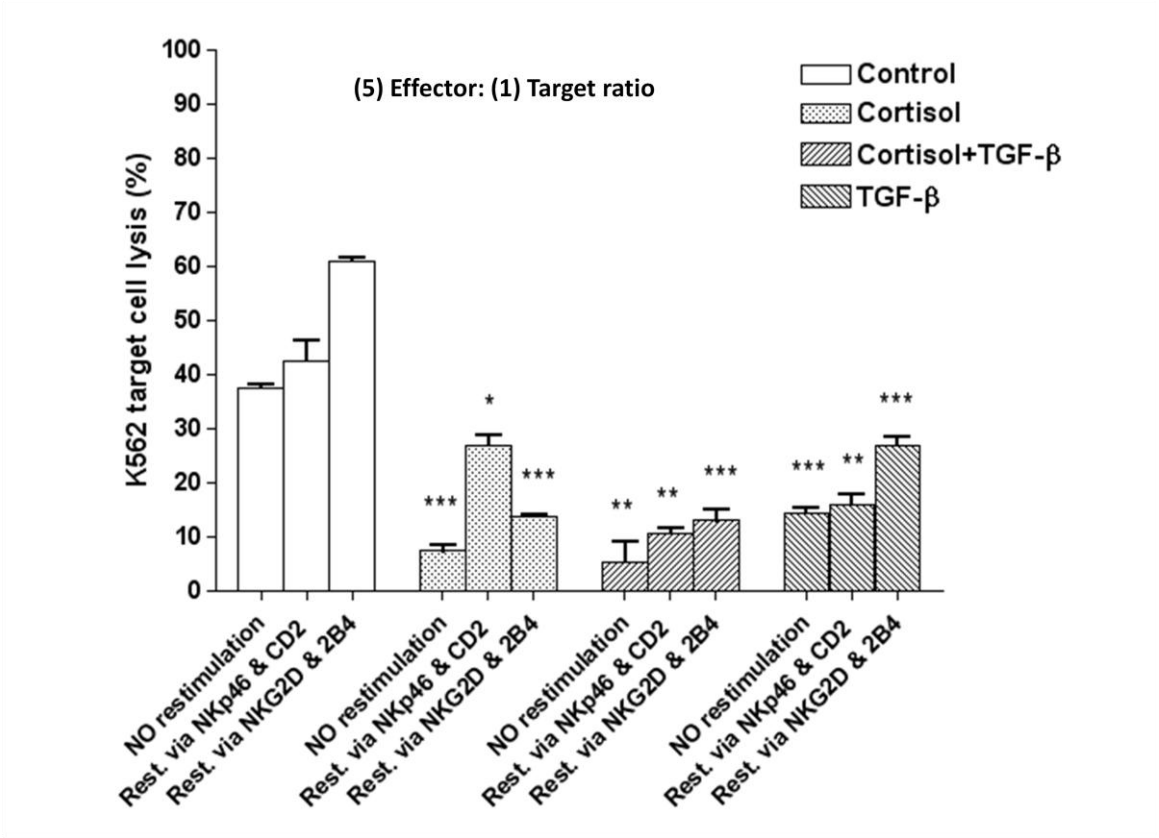


Figure (48): Restimulation experiment with NK cells that had been pretreated with cortisol or TGF-β1. On day 8 of their preparation, polyclonal NK cells were either left untreated or treated for 72 h with 5µM cortisol or 5ng/ml TGF-β1 or both. Afterwards, NK cells were co-cultured for further 48 h with MACSiBeads loaded with NKp46 and CD2 antibodies or on plates that had been coated with anti-NKG2D and anti-2B4 antibodies or without further stimulation as control. NK cells were then added in triplicates at the indicated E(ffector): T(arget) ratio to luciferase-transfected K562 (K562-fluc) target cells (A: E:T ratio 10:1, B: E:T ratio 5:1). Luciferase activity was measured after 72 h. A representative experiment (n=3) is shown.P values were determined by unpaired student t-test using Graphpad Prism 4.

4 Discussion

Cancer immunotherapy has long been considered as an interesting concept for tumour treatment. For ovarian cancer there is clear evidence that immunological parameters are crucial for the clinical outcome: While infiltration of a tumour with cytotoxic T cells is a good prognostic sign (Zhang et al., 2003), an abundance of regulatory T cells correlates with poor survival (Curiel et al., 2004). This suggests that the bolstering of anti-tumour immune responses (Mantia-Smaldone et al., 2012) could provide a clinically meaningful benefit. Nevertheless, an attempted immunotherapy with adoptive transfer of genetically engineered T cells failed for ovarian cancer patients since the infused T cells neither infiltrated the tumour nor persisted *in vivo* (Kershaw et al., 2006). Likewise, antibody-mediated immunotherapy which has been very successful for other malignancies (Slamon et al., 2001; Weiner et al., 2010) has been ineffective in ovarian cancer yet (Berek et al., 2009). Some clinically meaningful responses were at least achieved in a trial that aims at inducing immunity against the tumour antigen NY-ESO1 (Odunsi et al., 2012). Multi-peptide vaccination against a set of 8-20 individually determined tumour antigens was found to be highly beneficial for renal cell carcinoma (Walter et al., 2012) and colorectal cancer (poster presented at ASCO 2012). This approach is currently being tested on a first ovarian cancer patient in our clinics (ongoing experimental treatment). Thus, some progress is being made with regard to the optimal mode of activating the immune system against ovarian cancer.

Part of the problem, however, seems to be that ovarian cancer deploys multiple mechanisms to suppress immunity in the tumour microenvironment (Curiel et al., 2003; Hemminki et al., 2003; Kryczek et al., 2006; Qian et al., 2009; Yigit et al., 2010; Krempski et al., 2011). Accordingly, a better understanding of immunosuppression in the tumour microenvironment will be needed to develop more effective immunotherapies. In this context, promising animal data were obtained by targeting of tumour-associated macrophages (Galmbacher et al., 2010). Attempts to overcome negative regulation of immune responses on humans have also been positively evaluated in cancer patients. The antibody-mediated blockade of CTL-A4 on lymphocytes was found to be effective enough in melanoma patients to allow the approval of the respective antibody ipilimumab (Robert et al., 2011) in spite of considerable treatment-related autoimmunity. With this same antibody, promising early clinical data were also obtained for ovarian cancer patients (Hodi et al., 2008). A slightly more tumour-specific approach would be the targeting of interactions between PD-1 on immune cells and PD-L1 on

tumour or accessory cells. Again, clinical data obtained on other diseases look promising (Topalian et al., 2012; Brahmer et al., 2012). Thus, the field of cancer immunotherapy seems to be rapidly evolving and the concept that immunosuppressive signals must be overcome in order to achieve the desired response is getting recognized.

In this context, we have investigated 11β-HSD1 as a potential mediator of immune escape in ovarian cancer.

11β-HSD1 is an oxido-reductase that is essential for the conversion of biologically inactive cortisone into active cortisol. Together with its counterpart 11β-HSD2 (which *in vivo* catalyzes the opposite reaction) 11β-HSD1 regulates the local bioavailability of cortisol. In ovarian surface epithelial cells, 11β-HSD1 induces a local anti-inflammatory environment in response to the ovulation-inducing increase in luteinizing hormone ("LH surge") (Yong et al., 2002; Rae et al. 2004). Since ovulation causes massive tissue damage which triggers the production of pro-inflammatory cytokines, induction of 11β-HSD1 and the resulting increase in local cortisol levels limit inflammatory responses in the ovarian surface epithelium. Importantly, there is epidemiological evidence that inflammation and the ovulation-associated injury-repair processes contribute to the development of ovarian cancer (Yong et al., 2002; Modan et al., 2001). Accordingly, there was a clear biological rationale for investigating a potential expression of the anti-inflammatory enzyme 11β-HSD1 in ovarian cancer tissues. In fact, immunohistochemical stainings revealed of 11β-HSD1 expression in 20/20 tumours with expression levels mostly exceeding that of human liver (used as positive control). Healthy tissues, in contrast, showed low or undetectable amounts of 11β-HSD1. 11β-HSD2, on the other hand, was hardly detectable in human ovarian cancer tissues whereas clear stainings were obtained with tissue sections from placenta (used as positive control). Thus, the equilibrium between the two enzymes that catalyze either the activation or the inactivation of cortisol was clearly on the side of the anti-inflammatory enzyme 11β-HSD1.

Interstingly, we found the highest 11β-HSD1 mRNA and protein expression in primary human ovarian cancer tissues, but not in cell lines or purified EpCAM-positive tumour cells from patient material. We thus performed immunofluorescent double stainings on primary human ovarian cancer tissues (serous-papillary, psammomatus -serous, endometrioid ovarian carcinomas and borderline tumours). These revealed a co-localization of 11β-HSD1 with markers expressed by tumour-infiltrating myeloid-derived suppressor cells (MDSC) or

tumour-associated macrophages (TAM) (CD14, CD68, and CD85) but not with the tumour cell marker EpCAM. Similarly high levels of 11β-HSD1 were detected in macrophages and dendritic cells (Thieringer et al., 2001; Zhang et al., 2005; Freeman et al., 2005). Thus, MDSC or TAMs are the cell types that are most likely responsible for conversion of biologically inactive cortisone into active cortisol in ovarian cancer. Functionally, this would represent a new mechanism of immunosuppression in the tumour microenvironment.

We found that over-expression of 11β-HSD1 in ovarian cancer correlated with elevated cortisol levels in serum, ascites and tissue exudates from ovarian cancer patients as compared to healthy controls (serum) or patients with primary peritoneal carcinoma (ascites). This is in line with some other studies in which high levels of cortisol were detected in blood, saliva and ascites from ovarian cancer patients (Touitou et al., 1996; Lutgendorf et al., 2008; Weinrib et al., 2010). We thus hypothesized that local activation of endogenous GC could impair anti-tumour immune responses and thereby prevent a successful immunotherapy. Described effects of GC on the actual tumour cells, in contrast, seem to suppress rather than promote tumour growth.

Nevertheless, we also explored effects of cortisol on ovarian cancer cells. On protein level, we found GR to be highly expressed in primary human ovarian cancer tissues, as well as in ovarian cancer cell lines. Accordingly, cortisol binding could have a direct effect on ovarian cancer cells. *In vitro* tests, however, did not show any effect of GC on tumour cell proliferation or viability.

As human testing is limited, we then decided to address the immunomodulatory role of endogenous GC in in an inducible mouse model for ovarian cancer. Tumours grown in $PTEN^{loxP/loxP}$; loxP-Stop-loxP-$kras^{G12D}$ mice (Dinulescu et al., 2006) display a histomorphology and a metastatic pattern that closely resemble human ovarian cancer of the endometrioid subtype. In addition, the 11β-HSD1 expression pattern corresponds to that found in the human disease.

To determine the effect of GC on the spontaneous immune response in this tumour model, $PTEN^{loxP/loxP}$; loxP-Stop-loxP-$kras^{G12D}$ mice were adoptively transferred with $CD90.2^{-}$ hematopoietic stem cells isolated from bone marrow of either Lck-Cre $GR^{loxP/loxP}$ mice (T-cell GR-deficient mice) or $GR^{loxP/loxP}$ mice (reconstituted control). CD45.1 (Ly5.1) expressing

donor mice were used as additional controls to assess successful immunological reconstitution. Once the immune systems of the reconstituted mice had been restored, tumour development was initiated by injection of adenoviral Cre-recombinase into the ovarian bursa and the tumours were allowed to grow until the animals developed symptoms and had to be sacrificed. Surprisingly, this showed a shorter survival of those mice that had been reconstituted with GC-insensitive T cells. Immunohistochemical investigation of the explanted tumours nevertheless revealed that the T cell-specific GR knock-out had enabled improved intratumoral infiltration with T cells and DCs as compared to control mice. In GR-deficient mice, however, the majority of infiltrating T cells displayed a $Foxp3^+$ (regulatory) phenotype which might explain the poor survival of these mice. Along the same line, *ex vivo* studies observed that recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege, poor prognosis and predicts reduced survival (Curiel et al., 2004; Wolf et al., 2005). Furthermore, plasmacytoid dendritic cells infiltrating ovarian cancer constitute an independent prognostic factor associated with early relapse (Labidi-Galy et al., 2012). Moreover, while the mice with a T-cell specific GR-deficiency showed increased autoimmune response in antigen-induced arthritis (Baschant et al., 2011), a very recent report describes that thymocyte responsiveness to endogenous GC is required for immunological fitness and robust adaptive immune responses. According to this source, thymocyte responsiveness to GC signaling promotes the selection of T cells that have sufficient affinity for self, whereas otherwise (in lck-Cre $GR^{loxP/loxP}$-mice) an immunocompromised state might occur (Mittelstadt et al., 2012). Thus, impaired functionality of T cells or differentiation into T_{reg} or both may have contributed to the unexpected outcome.

To better understand the *in vivo* interactions between tumours and immune cells in these mice, messenger RNA (mRNA) was extracted from explanted tumours and analyzed for various pro-inflammatory and angiogenic markers. 11β-HSD1, GR, IL-1β, TNF-α and MMP-9 mRNA are highly expressed in OvCA from mice that had been reconstituted with Lck-Cre $GR^{loxP/loxP}$ hematopoietic cells as compared to tumours from mice that had received $GR^{loxP/loxP}$ cells and healthy ovaries from control mice , whereas only IL-1α,) mRNA showed the higher expression levels in tumours from mice with GC-sensitive lymphocytes. Healthy ovaries from control mice were again used as controls. These findings are in line with several studies which show that high expression of matrix metalloproteinases (MMP), key proteins required for tumour cell penetration of the extracellular matrix, promote overall tumour progression (Sood et al., 2006). Also, it appears likely that the high expression of 11β-HSD1 in OvCA tissue is a

response to the abundant pro-inflammatory cytokines like IL-1α (Yong et al., 2002; Chapman et al., 2006), IL-1β (Escher et al., 1997; (Cai et al., 2001; Tomlinson et al., 2001; Li et al., 2006; Chapman et al., 2006) and TNFα (Escher et al., 1997; Thieringer et al., 2001; Cai et al., 2001; Tomlinson et al., 2001; Chapman et al., 2006).

This experiment showed that the use of T cells that had been rendered insensitive to GCs could improve immune cell infiltration into the tumour microenvironment and thus overcome a major obstacle in cancer immunotherapy (Rosenberg, 2008). Vice versa, local activation of cortisol could be a further "road signs" that keep T cells out of the tumour microenvironment in ovarian cancer (Bagnato et al., 2005; Buckanovich et al., 2008; Schaer et al., 2011; Molon et al., 2011). Nevertheless, while this would normally be a desirable outcome (Fridman et al., 2012), it appears that tumour-infiltrating T cells were rapidly tolerized (Bai et al., 2008) and thus acted in favor of the tumour rather than of the host. Accordingly, inhibition of GC signaling would have to be combined with vaccination or with antagonisation of further tolerogenic mechanisms in the tumour microenvironment. Unfortunately, however, a virus infection in the animal facility precluded further experiments in this direction.

We thus decided to explore possibilities for initiating an anti-tumour immune response in the presence of glucocorticoids and TGF-β which is also known to be abundant in the tumour microenvironment. GCs and TGF-β mutually contribute to the limitation and/or termination of inflammatory processes and are both involved in the regulation of cell growth, development and differentiation (Periyasamy and Sánchez, 2002; Peltier et al., 2003). Moreover, GCs and TGF-β modulate each other's activities (Periyasamy and Sánchez, 2002; Peltier et al., 2003). High levels of TGF-β have been detected in many tumours including ovarian cancer (Hirte & Clark 1991; Zeimet et al., 1998; Nash et al., 1999; Abendstein et al., 2000; Santin et al., 2001). Among all cytokines, TGF-β possesses the strongest immune-inhibitory properties. Nevertheless, its effect on immune cells is sometimes reversible (Wilson et al., 2011). High levels of cortisol have already been demonstrated in this thesis and were also detected by others in ovarian cancer ascitic fluid (Touitou et al., 1996; Lutgendorf et al., 2008; Weinrib et al., 2010) and GCs exert anti-inflammatory and immune suppressive effects on immune cells (Sternberg, 2006). In addition, glucocorticoids are often administered as a co-treatment with chemotherapy to reduce chemotherapy-related side effects (Runnebaum and Brüning, 2005; Zhang et al., 2006; Sui et al., 2006; Melhem et al., 2009; Chen et al., 2010).

Thus, both GCs and TGF-β likely contribute to immunosuppresion in ovarian cancer patients and both must be overcome in order to prevent immune escape.

In this context, we placed a special focus on natural killer (NK) cells. NK cells constitute a vital part of the innate immune system and play a major role in the rapid immune responses against tumour cells and virus-infected cells (Kiessling et al., 1975; Herberman et al., 1975; Riccardi et al., 1980; Ljunggren and Karre, 1985; Natuk and Welsh 1987; Salazar-Mather et al., 1998; Biron et al., 1999; Janeway, 5th Edition, 2005; Lodoen and Lanier; 2006). Moreover, NK cells have been reported to play a central role in controlling tumour promotion, growth and metastasis in rodents and humans (Smyth et al., 2002) via release of immune-stimulatory cytokines and cytolytic granules (Smyth et al., 2002). NK cells may be able to deliver the "spark" to initiate a more potent immune response involving further cell types (Kelly et al., 2002). Accordingly, there is a strong rationale for inducing NK cell responses against tumours. We thus investigated whether specific signaling pathways in NK cells could still be activated in the presence of GC and/or TGF-β. To understand the influence of these factors on NK cell activation a careful investigation of the various activating NK cell receptors, including NKp30, NKp44, NKp46, DNAM-1, 2B4, CD2 and NKG2D, was carried out.

Flow cytometeric analysis revealed that cortisol decreases the expression of NKp30, NKp44, CD2 and NKG2D on NK cells while upregulating NKp46. Cortisol seems to have no effect on DNAM-1 or 2B4 expression. Similarly to cortisol, TGF-β1 decreases NKp30, CD2 and NKG2D expression levels on NK cells, but TGF-β1 can also downregulate DNAM-1. Interestingly, TGF-β1 does not affect expression of NKp46, NKp44, or 2B4. Cortisol and TGF-β1 act synergistically to further downregulate NKp30 and NKG2D receptors on NK cells. On mRNA level, the data revealed that cortisol decreases the expression of NKp30, CD2 and NKG2D on NK cells whereas only a slight down-regulation of NKp46 was observed. Also, Cortisol has no effect on DNAM-1and 2B4 mRNA expression, while NKp44 was not detected in cortisol-treated NK cells. Likewise, TGF-β1 decreases the expression of NKp30 and NKG2D, but has no effect on CD2, DNAM-1 and 2B4, whereas NKp46 was only slightly down-regulated. NKp44 was not detected in TGF-β1-treated NK cells. Cortisol and TGF-β1 were further found to act synergistically in downregulating NKG2D, NKp30 and CD2.

Several studies provided evidence that methylprednisolone treatment, which is commonly applied to patients suffering from inflammatory conditions, generally reduces the surface density of the main activating receptors involved in NK cytotoxic function, including NKp30, NKp44 and NKG2D. NKp46 expression on freshly isolated NK cells remained, in contrast, unaltered (Vitale et al., 2004; Chiossone et al., 2007). Another study reported that cortisol inhibited the surface expression of natural cytotoxicity receptors NKp46 and NKp30 on NK cells which correlated with a strongly reduced cytolytic activity of NK cells (Mavoungou et al., 2005). A similar phenomenon had been previously reported for TGF-β1 which strongly downregulates the expression of NKp30, NKG2D, DNAM-1 and 2B4 but not NKp46, leading to an impairment of NK cell effector functions (Castriconi et al., 2003; Friese et al., 2004; Robson et al., 2009; Carlsten et al., 2009; Wilson et al., 2011; Sun et al., 2012).

In the present study, the down-regulation of activating NK cell receptors in response to treatment with cortisol, TGF-β1 or both resulted in a highly significant reduction of NK cytolytic activity against K562 cells. Likewise, CD107a expression which is a degranulation marker for the cytolytic activity of activated NK cells (Peters et al., 1991; Fukuda, 1991; Alter et al., 2004) was down-regulated upon treatment with either cortisol or TGF-β1 and was further downregulated upon treatment with both. In a similar vein, other groups (Nair et al., 1984; Callewaert et al., 1991; Zhou et al., 1997; Vitale et al., 2004; Mavoungou et al., 2005) have reported that impairment of expression of NK-triggering receptors leads to reduced cytotoxicity against different tumour targets. Also the reduction of CD107a surface levels on TGF-β1- (Kopp et al., 2009; Wilson et al., 2011) or glucocorticoid-treated (Elftman et al., 2010) cytotoxic effector cells was in accordance with the previous literature.

A novel finding, however, was that surface expression of the activating NK cell receptor NKp46 can even by induced by cortisol treatment. Accordingly, we wondered whether NKp46 might keep up a certain NK cell functions even in individuals with high cortisol levels like ovarian cancer patients. Consequently, we investigated whether NK cells could be activated even in the presence of GCs via stimulation of NKp46. As a read-out, NK cell activity was assessed against K562 tumour cell targets. In line with this hypothesis, restimulation experiments with NK cell activation beads loaded with agonistic antibodies against NKp46 and CD2 revealed that the lytic activity of cortisol-treated NK cells could partially be restored. No rescue, however, was possible when via the triggering of NKG2D and 2B4 or when the cells had previously been exposed to TGF-β1.

Taken together, we have shown that endogenous glucocorticoids are abundant in ovarian cancer which is likely related to a high expression of the oxido-reductase 11β-HSD1 on myeloid cells since this enzyme converts cortisone into biologically active cortisol. In an inducible mouse model for ovarian cancer, adoptive transfer of glucocorticoid-insensitive hematopoietic cells from Lck-Cre $GR^{loxP/loxP}$ mice improved intratumoral T cell infiltration. Unfortunately, however, intratumoral T cells displayed a regulatory T cell phenotype which correlated with shortened survival. Still, synergy with therapies that rely on good intratumoral infiltration of highly activated T cells appears likely. In addition, immunomodulatory treatments that aim at a reduction of tumour-derived cortisol could benefit from the targeting of further tolerogenic factors in the tumour microenvironment (e.g. TGF-β). Ideally, the infiltrating T cells could then retain an effector phenotype and exert an anti-tumour effect.

In the second parrt of this thesis, *in vitro* experiments on primary human natural killer cells showed that both TGF-β1 and glucocorticoids strongly inhibit the majority of immune-activating pathways in these cells. NKp46, in contrast, gets even induced by glucocorticoids which suggest that this receptor serves to maintain a residual immune function in the presence of cortisol which could possibly be exploited for immunotherapy.

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6 List of Abbreviations and Acronyms

°C	Celsius
µg	microgram
µl	microlitre
µM	micromolar
11β-HSD1	11-beta hydroxysteroid dehydrogenase 1
11β-HSD2	11-beta hydroxysteroid dehydrogenase 2
2B4	natural killer cell receptor 2B4
aa	amino acid
ACD-A	sodium citrate solution
ADCC	antibody-dependent cellular cytotoxicity
AdCre	adenovirus encoded Cre-recombinase
AICL	activation-induced C-type lectin
AP-1	activator protein 1
APS	ammonium persulfate
BAT3	nuclear factor HLA-B associated transcript 3
bp	base pair
BSA	bovines serum albumin
CA-125	cancer antigen 125 or carbohydrate antigen 125
CBG	corticosteroid binding globulin
CD	cluster of differentiation
cDNA	complementary DNA
cIAP2	cellular inhibitor of apoptosis 2
cm^2	square centimeters
CMV	cytomegalovirus
CO 2	carbon dioxide
COX-2	cyclo-oxygenase-2
CT	cycle threshold
CTLs	cytotoxic T cells
DAB	diaminobenzidine
DCs	dendritic cells
dd H2O	double-distilled water
DHEA	dehydroepiandrosterone

DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EOC	epithelial Ovarian Cancer
EpCAM	epithelial cell adhesion molecule
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLuc	firefly luciferase
$Foxp3^+$	Forkhead box P3
GCs	glucocorticoids
gDNA	genomic DNA
GM-CSF	granulocyte–macrophage colony-stimulating factor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
Gy	Gray
h	hour
H60a	histocompatibility 60a
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSA	human serum albumin
IFN-γ	Interferon-gamma
IL	Interleukin
ITIM	immunoreceptor tyrosine-based inhibion motive
Kb	kilobase
kDa	kiloDalton
KIR	killer cell Ig-like receptors
KLR	killer cell lectin-like receptors
KLRK1	killer cell lectin-like receptor subfamily K, member 1 gene
K-ras	Kirsten rat sarcoma viral oncogene homolog protein

LAMP-1	lysosomal–associated membrane protein-1
LH	luteinizing hormone
LIA	luminescent immunoassay
mAbs	monoclonal antibodies
MACS	magnetic-activated cell sorting
MDSC	myeloid-derived suppressor cells
mg	milligram
MHC	major histocompatibility complex
MIC-A -B	MHC class I chain related -A -B
min	minute
ml	milliliters
MMP	matrix metalloproteinase
MR	mineralocorticoid receptor
mRNA	messenger RNA
MULT-1	murine UL16-binding protein like transcript
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NCRs	natural cytoxicity receptors
ng	nanogram
NK	natural killer cells
NKG2D	natural killer group 2D
NKT	natural killer T cells
nm	nanometer
NP-40	nonyl phenoxypolyethoxylethanol
NY-ESO-1	cancer-testis antigen
OD	optical Density
OSE	ovarian surface epithelial
OvCa	ovarian cancer
PBL	peripheral blood lymphocytes
PBMC	peripheral blood monocytes
PBS	phosphate buffered Saline
PBS-T	phosphate-buffered saline with 0.05% Tween20
PCR	polymerase chain reaction
PD-1	programmed cell death 1

PE	R-phycoerythrin
PFA	paraformaldehyde
PG	prostaglandins
PMA	phorbol 12-myristoyl 13-acetate
PMSF	phenylmethanesulfonylfluoride
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PVDF	polyvinylidene Fluoride Membrane
PVR	polio virus receptor
qRT-PCR	(semi-)quantitative real-time PCR
RAE1	RNA export 1 homolog
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute cell culture medium 1640
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulfate
SDS-PAGE	Sodium dodecylsulfate (SDS) - Polyacrylamide Gel Electrophoresis
SFI	specific fluorescence intensity
SHP	Src-homology protein
TAE	Tris-acetate-EDTA
TAM	tumor-associated macrophages
TBS	Tris-buffered saline
TEMED	tetramethylethylenediamine
TGF-β	transforming growth factor-beta
Th	helper T cells
TNF-α	tumor necrosis factor-alpha
Treg	regulatory T cells
ULBP	UL16 binding protein
UV	ultraviolet
V	Volt
VEGF	vascular endothelial growth factor
X g	gravity
γδ T	gamma delta T cells

CURRICULUM VITAE

PERSONAL DATA

- **Name** Ahmed Adel Seida
- **Date of birth** 22.03.1982
- **Place of birth** Giza, Egypt
- **Nationality** Egyptian

UNIVERSITY DEGREES

- **2006** Master degree in Veterinary Sciences from Microbiology and Immunology Department, Faculty of Veterinary Medicine, Cairo University, Egypt, thesis about "Preparation of Monoclonal Antibodies against Tetanus Toxin and evaluation of its neutralizing potentials".

- **2003** Bachelor degree in Veterinary Sciences ranked 4th in my class (A class of 756 students) from Faculty of Veterinary Medicine, Cairo University, Egypt.

CAREER HISTORY AND POSITIONS HELD

- **2009-Present**: PhD Student in Cancer Immunology at Interdisciplinary Center for Clinical Research (IZKF), junior research group "Tumor progression and immune escape", University of Würzburg, School of Medicine, Germany

- **2008:** (Marie Curie Scholarship) Guest student at Section of Immunogenetics, Faculty of Medicine, Rostock University, Germany, financed by the European Union as part of a Marie Curie Research Training Network for 8 months.

- **2006-present:** Senior Teaching/Research Assistant at Microbiology and Immunology Department, Faculty of Veterinary Medicine, Cairo University, Egypt.

- **2004-2006:** Teaching/Research assistant at Microbiology and Immunology Department, Faculty of Veterinary Medicine, Cairo University, Egypt.

- **2004-2006:** Research Assistant at Monoclonal Antibodies and Vaccines Unit in the Holding Company for Biological Products & Vaccines (VACSERA), Ministry of Health, Cairo, Egypt.

CONFERENCES, TRAINING COURSES AND WORKSHOPS

2012:

- 7th Annual Training Network Meeting of the graduates schools GK520 (Graduate collage Immunomodulation), GK1660 (Graduate collage Adaptive immunity) and SFB 685 (Graduate collage Immunotherapy), July 15-17, Kloster Schöntal (Germany), Poster presentation.
- 4th Network Meeting of German research council (DFG) graduates schools GK520 (Graduate collage Immunomodulation), GK592 (Graduate collage Lymphocytes) and GK794 (Graduate collage Cellular mechanisms of immune-associated processes), Nov 15-17, Kloster Schöntal (Germany), Poster presentation.
- 10th CIMT 2012 (Cancer Immunotherapy Meeting), May 23-25, Mainz (Germany), Poster presentation. (CIMT Scholarship).
- 11th ESID Prague Spring Meeting, Department of Immunology, 2nd Medical School, Charles University, University Hospital, May 14-15, Prague, (Czech Republic).(ESID Scholarship).
- 7th ENII EFIS-EJI Spring School in Advanced Immunology, April 15-22, Sardinia, (Italy). (ENII EFIS/EJI Scholarship).
- German Dermatological Research Association (ADF) -"Winter school of adaptive immunity", Jan 26-28, Zugspitze, (Germany). (ADF Scholarship).

2011:

- 2nd EFIS-EJI Intensive Educational Course in Clinical immunology (Advanced Course in Innovative Immunologically Based Therapies), Nov 28-30, Paris (France), Poster presentation. (EFIS-EJI Scholarship).
- The EFIS-EJI Ruggero Ceppellini Advanced School of Immunology "Innovative strategies to prevent transplant rejection", Oct 26-29, Sorrento, (Italy), Poster presentation. (EFIS-EJI Scholarship).

- 6th International Symposium organized by the students of the Graduate School of Life Sciences of the University of Würzburg, Oct 19-20, Würzburg, (Germany) Poster presentation and member of the student organizing committee.
- PIVAC-2011 "The Eleventh International Conference on Progress in Vaccination Against Cancer", Oct 10-13, Copenhagen, (Denmark), Poster presentation. (EFIS-EJI Scholarship).
- Summer School of Innate and Adaptive Immune System: Tool and Challenge for Gene Therapy, Center for Molecular Medicine, University of Cologne, September 14-15, Cologne, Germany.
- 16th EFIS-EJI Symposium IMPULSE 2011 (IMmune-related Pathologies: Understanding Leukocyte Signaling and Emerging therapies), September 3-7 Visegrad, (Hungary), Poster presentation. (EFIS-EJI Scholarship).
- 6th Annual Training Network Meeting of the graduates schools GK520 (Graduate collage Immunomodulation), GK1660 (Graduate collage Adaptive immunity) and SFB 685 (Graduate collage Immunotherapy), June 6-8, Klosterhospiz Nereshiem (Germany), Poster presentation.
- Frontiers in Tumor Immunology: Innovative Immunotherapies, May 02 - 03, University of Tübingen, (Germany), Poster presentation.
- 10th Charles Rodolphe Brupbacher Symposium (Cancer Genome and DNA Repair), Feb 16-18, University Hospital Zurich, (Switzerland), Poster presentation.

2010:

- 1st EFIS-EJI Intensive Educational Course in Clinical immunology (Advanced Course in Innovative Immunologically Based Therapies), Dec 1-4, Paris (France). (EFIS-EJI Scholarship).
- International workshop of the German research council (DFG) transregional research center TR52 (Transcriptional programming in the immune system), Nov17-20, Würzburg (Germany).
- 5th Network Meeting of the German research council (DFG) graduates schools GK520 (Graduate collage Immunomodulation), GK1660 (Graduate collage Adaptive immunity) and GK794 (Graduate collage Cellular mechanisms of immune-associated processes), Nov 7-9, Kloster Schöntal (Germany), Oral presentation.

- Hannover Summer School “Gene regulation, cell differentiation and tolerance”, September 12th – 16th, Goslar, Hessenkopf (Germany).
- 9th Bern International Summer School on "Inflammation and Apoptosis", Aug 8-10, Kandersteg (Switzerland), Poster presentation.
- Animal rights course which organized by (Federation of European Laboratory Animal Science Associations), July 26-30, Würzburg (Germany).
- Annual meeting of the clinical research group KFO 124 (The Tumour Microenvironment: Target and Immune Modulator of the Immune Response, July 16, Würzburg (Germany).

2009:

- 4th Network Meeting of German research council (DFG) graduates schools GK520 (Graduate collage Immunomodulation), GK592 (Graduate collage Lymphocytes) and GK794 (Graduate collage Cellular mechanisms of immune-associated processes), Nov 15-17, Kloster Schöntal (Germany), Poster presentation.
- International Leopoldina Symposium on (Evolution of Programmed Cell Death in Infection and Immunity), September 18-20, Würzburg (Germany).

2005:

- Ten days course on Monoclonal Antibodies Production (Protocols & Know – How) in the holding company for biological products & Vaccines (VACSERA), Funded from Cairo University, Cairo (Egypt).
- Six days course "Molecular Diagnostic Microbiology", July 27- Aug 2, Intervet company and Faculty of veterinary medicine Cairo University, Funded from Cairo University, Cairo (Egypt).

2004:

- Member of the organizing committee of the "First International Conference of Scientific Research and its Applications", Aug 18-20, Cairo (Egypt).

PUBLICATIONS

- Häusler SF, Montalbán Del Barrio I, Strohschein J, Anoop Chandran P, Engel JB, Hönig A, Ossadnik M, Horn E, Fischer B, Krockenberger M, Heuer S, Seida AA, Junker M, Kneitz H, Kloor D, Klotz KN, Dietl J, Wischhusen J.: Ectonucleotidases CD39 and CD73 on OvCA cells are potent adenosine-generating enzymes responsible for adenosine receptor 2A-dependent suppression of T cell function and NK cell cytotoxicity. Cancer Immunol Immunother (2011) 60:1405–1418