# Genetical, structural and functional characterization of the human BTNL gene cluster

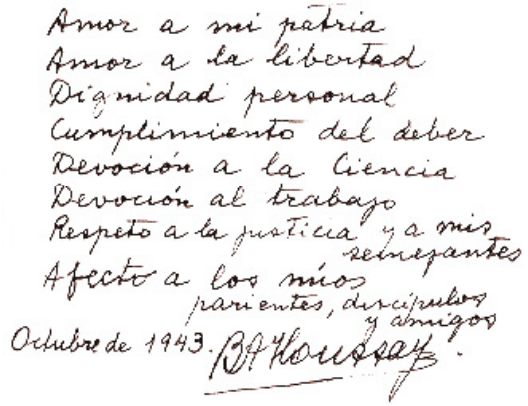
Johanna Aigner

THESIS DOCTORAL/ 2013

XAVIER ESTIVILL

BIOINFORMATICS AND GENOMICS

upf. Universitat Pompeu Fabra Barcelona


Amor a mi patria
Amor a la libertad
Dignidad personal
Cumplimiento del deber
Devoción a la Ciencia
Devoción al trabajo
Respeto a la justicia y a mis semejantes
Afecto a los míos parientes, discípulos y amigos
Octubre de 1943. B. Houssay

*Für meine Eltern*.

# Acknowledgements

Firstly, I am very grateful to my supervisor **Xavier**, for giving me the chance to work in his laboratory. Thank you for all the invested time in the supervision of my thesis and for your readiness for scientific discussion. In addition, thank you for being extremly patient and and helpful during every step of my graduate career.

I would like to also thank **Eulàlia** for kindly proofreading my manuscripts and giving me helpful comments on my thesis project. Thank you for always finding the time to see me when I needed advice.

Millions of thanks to **Elisabet**, **Elena** and **Esther** for all the great time we spent together. Not only did you help me with my projects but also have we spent a lot of memorable times outside work. You were my family in Barcelona.

Many thanks go to **Sergi**, who developed the genotyping assay, to **Kely** who did the SNP analysis, to **Lluis** who helped with the microarray analysis.

Especially I wanna thank our technicans. **Marta** for keeping the lab running, **Birgit** for her technical assistence in so many experiments and **Anna** for her help with the genotyping.

Throughout the five years, I have been fortunate to have extraordinary colleagues who were always helpful and created a nice working atmosphere in the laboratory. Thanks to **Elisa**, **Susa**, **Laia**, **Ester**, **Anna**, **Mónica**, **Georgia**, **Nadia**, **Mariona**, **Marc**, **Charlotte**, **Hyun**, **Ana**, **Daniel**, **Laura**, **Joan** and **Aparna**.

A sepial mention goes to “the family” (**Tony**, **Kiana**, **Tobias**, **Almer** and **Raik**) for all the funny and great moments we spent. You always had an open ear and cheered me up in difficult moments. I will miss the cañas with you guys.

I can hardly imagine a better PhD program than the one at the CRG and I am deeply thankful to the CRG Committee of Graduate Studies for caring so well for us.

In these lines I want to thank my family for their motivation, trust, love and for always supporting me (Vielen Dank **Mama**, **Papa** und **David** für eure Unterstützung und das ihr immer an mich geglaubt habt). This thesis is dedicated to them.

# Abstract

In this thesis, we undertook a broad genomic, evolutionary, transcriptomic and functional analysis of a cluster containing three BTNL genes, namely *BTNL8*, *BTNL3* and *BTNL9*, located on human chromosome 5q35.3.

In the first chapter we report the identification of a 56 kb deletion copy number variant (CNV), which results in the formation of a novel chimeric gene, *BTNL8*3*, and leads to an upregulation in the expression-level of the third gene in the cluster, *BTNL9*. Next, we developed a genotyping assay and undertook a population analysis of this variant in several Hap Map and human diversity panel (HGDP-CEPH) populations. With this genotyping assay we could identify clear differences in the stratification of the *BTNL8_BTNL3-del* allele amongst major continental ethnic groups. In addition we report tagging SNPs in several population, facilitating the genotyping process of the *BTNL8-BTNL3* deletion variant in the future. Moreover, we show an influence of the deletion CNV in the expression-level of several genes involved in cancer and immune response, suggesting an involvement of this CNV in specific biological pathways.

In the second chapter we look for functional consequences of this CNV and found an upregulation of *BTNL9* in acute lymphoblastic leukemia (ALL) after glucocorticoid (GC) treatment. Previously, it was shown that high-level *BTNL9* correlates with high-risk in *MLL-AF4* rearranged acute lymphoblastic leukemia (ALL) patients. To check whether this might be due to in involvement of BTNL9 in GC-induced apoptosis, we analyzed several pre-B ALL cell-lines and found a clear correlation between *BTNL9* expression-level and resistance to GC in MLL rearranged ALL and at a lower level in *MLL* germ-line ALL. These results suggest a completely new and unexpected role for a BTNL protein and may led to the development of specific BTNL9 inhibitors to improve outcome of MLL rearranged ALL.

Overall, we provide a comprehensive analysis of a BTNL gene cluster. We identified a new *BTNL8*3* fusion-gene with potential implication in genetic pathways involved in immune regulation and proliferation, and show a clear function for BTNL9 in GC-resistance in *MLL* rearranged leukemia. This knowledge sheds more light on the BTNL family and may provide the basis for novel approaches using BTNL9 in MLL rearranged ALL therapy.

# Resum

En aquesta tesi, hem dut a terme un ampli anàlisi genòmic, evolucionari, transcriptòmic i funcional d'un clúster de tres gens (*BTNL8, BTNL3* i *BTNL9*) localitzats en el cromosoma humà 5q35.3.

En el primer capítol, presentem la identificació d'una deleció d'una variant en nombre de còpia (CNV en anglès) de 56 kb donant com a producte un nou gen quimèric (*BTNL8*3)*. Aquesta deleció és responsable de la sobre-expressió del tercer gen del clúster, *BTNL9*. Posteriorment, es desenvolupà un assaig de genotipació i es va dur a terme un anàlisi poblacional d'aquesta variant en mostres de diferents poblacions pertanyents al HapMap i el panell de diversitat humana (HGDP-CEPH). Aquest assaig de genotipació ens va permetre identificar clares diferències en l'estratificació de l'al·lel *BTNL8_BTNL3-del* entre grups continentals majors. A més, presentem tagging SNPs en diverses poblacions, facilitant una genotipació futura de la variant de deleció *BTNL8-BTNL3*. Finalment mostrem la influència de la deleció CNV en els nivells d'expressió de diferents gens involucrats en càncer i en la resposta immune, suggerint la involucració d'aquesta CNV en rutes biològiques específiques.

En el segon capítol d'aquesta tesi s'investiguen les conseqüències funcionals de la CNV trobant una sobre-expressió de *BTNL9* en leucèmia limfoblàstica aguda (ALL en anglès) després del subministrament de glucocorticoides (GC). S'havia mostrat ja prèviament que uns nivells elevats de *BTNL9* correlacionen amb un elevat risc en pacients de ALL amb reorganització de *MLL-AF4*. Per comprovar si aquesta observació és deguda a la implicació de BTNL9 en apoptosi induïda per GC, es varen analitzar diferents línies cel·lulars pre-B ALL trobant-se una clara correlació entre els nivells d'expressió de *BTNL9* i resistència a GC en ALL amb reorganització de *MLL* i nivells més baixos en *MLL* en ALL germinal. Aquests resultats suggereixen un paper completament nou i inesperat de la proteïna BTNL que podrien resultar en el desenvolupament de inhibidors específics de BTNL9 per millorar la prognosi de ALL amb reorganització de *MLL*.

En resum, en aquesta tesi proporcionem un anàlisi del clúster de gens humà *BTNL*. Identifiquem un nou gen de fusió *BTNL8*3* amb implicacions potencials en rutes genètiques involucrades en la regulació i proliferació immune i mostrem una clara funció de BTNL9 en la resistència a GC el la leucèmia amb reorganització de *MLL*. Aquestes observacions proporcionen un nou coneixement sobre la família de gens *BTNL* i podria proporcionar la base per noves teràpies basades en BTNL9 en ALL amb reorganització de *MLL*.

## Preface

The B7 family of protein is widely accepted to play an important role in inflammatory processes by altering T cell responsiveness. Through binding to their receptors on T cells these proteins are able to promote (e.g., B7-1, B7-2, ICOS-L) or inhibit (e.g., PD-L1, PD-L2, B7-H3, B7x) T cell activation, proliferation, maturation and cytokine production. In addition, several members have been identified to be expressed on different types of tumors as within the tumor microenvironment. Due to the immunosuppressive capacities of several B7 family members, aberrant expression of these molecules is thought to negatively interfere with the host immune response, leading to disease progression. Indeed, expression of B7 family proteins in many hematologic malignancies is often associated with poor prognosis and aggressive behavior of tumors. Currently, several B7 family members, such as CTLA-1 and PD-1 pathway inhibiting molecules are targeted in the treatment of cancer and recently, the first B7 pathway-targeting agent, anti-CTLA-4 mAb (ipilimumab) has been approved by the Food and Drug Administration (FDA) for the treatment of metastatic melanoma. In addition, ongoing studies targeting more recently described members of the family: B7-H3, B7x, B7-H6 are promising, however further clarification of their pathogenic role in hematologic malignancies will help to identify their most active role as immune adjuvants to conventional therapy.

However, regardless the tremendous progress in this field, up to this date little is known about the closely related butyrophilin-like (BTNL) proteins. The butyrophilin (BTN) family shares structural homology with B7 family members and similar to B7 proteins, almost all BTNs/BTNLs studied so far, have been shown to be able to dampen immune-response by negatively co-stimulating T cell activation, making them very interesting candidates in anti-tumor immunity. Consequently, in this study, we characterize a cluster containing three BTNL genes, located on human chromosome 5q35.3, at the genomic, transcriptional and functional level to gain more insight in the function of the BTNL proteins.

## Content

# INTRODUCTION

## 1.1 The B7 protein family

The B7-CD28 family consists of structurally related cell-surface proteins that have been shown to play key roles in pathways regulating T-cell activation and effector cell differentiation. These pathways can either contribute critical positive signals that promote and sustain T-cell responses, or they can provide critical negative signals that downregulate T-cell responses [1].

Two simultaneous but independent signals are used by macrophages and dendridic cells (DCs) to activate naive T-lymphocytes. The first signal is initiated from the antigen-specific T-cell receptors (TCR) interacting with an antigenic peptide presented by the major histocompatibility complex (MHC) class II on professional antigen-presenting cells (APC). However, the MHC II binding itself is insufficient to produce a T-cell response. In fact, lack of further stimulatory signals results in the induction of T-cell tolerance, called *anergy*. Therefore, a second signal is required, known as *co-stimulation* [1].

The most important co-stimulatory signal necessary to continue the immune response comes from B7-CD28 interactions. The B7 (B7-1 or CD80 and B7-2 or CD86) protein is present on the APC surface, and it interacts with the CD28 receptor on the T-cell surface [2]. This interaction leads to proliferation and cytokine production, promote cell survival and enhance expression of CD40 ligand (CD40L) and adhesion molecules necessary for trafficking, such as very late antigen-4 (VLA-4) [3]. Consistently, mice deficient in CD28 or both of its ligands (B7-1 and B7-2) have been shown to be severely impaired in $CD4^+$ T-cell proliferation [4]. However, the B7 pathway not only provides positive second signals but also contributes critical negative second signals that counteract T-cell activation by limiting, terminating, and/or attenuating T-cell responses Therefore, B7-1 and B7-2 bind to an inhibitory receptor, cytotoxic T-lymphocyte antigen-4 (CTLA-4 or CD152), which inhibits T-cell response and crucially controls peripheral T-cell tolerance and autoimmunity.

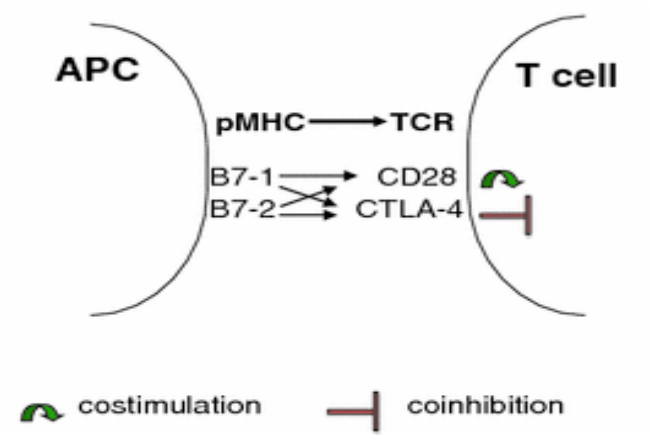



**Figure 1. Co-stimulatory and co-inhibitory function of B7-1/2 :CD28/CTLA-4 interaction.** APC, antigen-presenting cell; pMHC, peptide major histocompartibility complex; TRC, T-cell receptor; *From Seliger et al., 2012* [5]

## 1.1.2 Members of the B7 protein family

Over the past decade, several ligands and their counter-receptors with homology to B7-1 and B7-2 have been identified. These B7 homologues (B7-H') include: programmed death-1 ligand PD-L1 (B7-H1 or CD274), inducible co-stimulator ligand ICOS-L (B7-H2 or CD275), B7-H3 or CD276, B7x (B7-H4 or B7-S1), and programmed death-2 ligand PD-L2 (B7-DC or CD273) [6]. All members of the B7 family are transmembrane or glycosylphosphatidylinositol (GPI)-linked proteins characterized by extracellular IgV (variable) and IgC (constant) domains related to the variable and constant domains of immunoglobulin [7].

**Table 1. Nomenclature of B7 family molecules** *From Greaves and Gibben, 2013* [8]

| CD designation | Molecule | "B7" designation | Eponyms | "Common" name | Major ligands |
|---|---|---|---|---|---|
| CD80 | B7-1 | B7-1 | B7 | CD80 | CD28, CD152 |
| CD86 | B7-2 | B7-2 | — | CD86 | CD28, CD152 |
| CD274 | Programmed cell death ligand 1 | B7-H1 | PD-L1 | PD-L1 | CD279 (PD-1) |
| CD275 | Inducible co-stimulator ligand | B7-H2 | B7RP-1, B7h, | ICOS-L | CD278 (ICOS) |
| CD276 | B7 homologue 3 | B7-H3 | — | — | ??TLT-2 |
| | B7 homologue 4 | B7-H4 | B7S1, B7x | — | ??BTLA |
| | B7 homologue 6 | B7-H6 | — | — | NKp30 |
| CD273 | Programmed cell death ligand 2 | B7-DC | PD-L2 | PD-L2 | CD279 |
| CD277 | Butyrophilin SF3 | — | BT3.1, BTF5 | — | ? |

The CD designation is used preferentially in this review.

—Indicates no established designation in this category; ?, unknown; ??, evidence is contested or based on limited date.

According to their functions, the B7 family members are classified into three groups.

*Group I B7 molecules: B7-1/B7-2:CD28/CTLA-4 and ICOS-L:ICOS*

B7-1 and B7-2 are inducibly expressed on APC and other hematopoietic cells. B7-1 and B7-2 bind to the same receptors, stimulatory receptor CD28 and inhibitory receptor CTLA-4. CD28 is constitutively expressed on resting T-cells, and engagement of B7-1 or B7-2 with CD28 provides a vital positive signal, that is required for the activation, proliferation and maturation of naïve effector T-

lymphocytes ($T_{effs}$) by inducing production of interleukin-2 (IL-2) and anti-apoptotic factors [9]. However, CD28 does not affect T-cell activation unless the TCR is first engaged by cognate antigen. By contrast, CTLA-4 appears on the surface of T-cells only following their activation and binds B7-1 or B7-2 with a much higher affinity (50-200 fold higher) compared to CD28 [10]. The interaction of CTLA-4 with B7-1 and B7-2 delivers a negative or inhibitory signal to reduce T-cell activation [11]. This role of CTLA-4 as a negative regulator was clearly shown in CTLA-4-deficient mice, which display polyclonal T-cell activation and lymphoproliferative disorder that results in neonatal lethality [12]. In addition to regulating the activation of $T_{eff}$-cells, CTLA-4 plays a critical role in induction of peripheral tolerance [13]. In addition, some recent additional publications have implicated a major role for CTLA-4 in the downregulation of helper T(Th)-cell activity and enhancement of $FOXP3^+CD4^+CD25^+$ regulatory T-cell ($T_{reg}$) immunosuppressive activity [14, 15]. FOXP3 represses IL-2 transcription and upregulates expression of CTLA-4, thus $FOXP3^+CD4^+CD25^+$ $T_{reg}$-cells constitutively express CTLA-4 [16].

ICOS-L is expressed on B-cells, macrophages and DC but can also be detected on fibroblasts, epithelial cells and endothelial cells. ICOS-L serves as a ligand for ICOS another CD28 family molecule. ICOS is present on activated T-cells and B-cells and provides a positive stimulatory effect [17]. $ICOS^+$ T-cells have been shown to be involved in transplant rejection [18] as well as autoimmune responses [19] [20]. ICOS is thought to play a role in maintaining durable immune reactions and is expressed at particularly high levels in germinal center T-cells of follicular helper (TFH) cells. Mutations in the human ICOS gene result in an attenuated adult-onset common variable immunodeficiency (CVID), likely arising from loss of TFH cell function. This disease manifests with a variety of autoimmune phenomena as well as cancer and infection susceptibility [21].

CD28 and ICOS pathways have a synergistic function and deficiencies in both pathways led to complete T-cell tolerance *in vivo* and *in vitro* [22].

*Group II B7 molecules: PD-L1/PD-L2:PD-1*

PD-L1 mRNA is broadly expressed in different mouse and human tissues although its constitutive protein distribution is limited to a fraction of hematopoietic cells and some parenchymal cells. However, most normal tissue cells seem to be able to upregulate PD-L1 in the presence of strong inflammatory signals [23-26]. This broad distribution of PD-L1 suggests that it may regulate immune responses in both, lymphoid and non-lymphoid organs. Moreover, PD-L1 is aberrantly expressed by numerous human tumors, indicating that its protein expression is controlled by post-translational mechanism. This could be proinflammatory cytokines such as INF-$\gamma$ or loss of tumor-suppressors, including phosphatase and tensin homolog (PTEN) [27]. In contrast to PD-L1, PD-L2 protein expression is restricted to DC and macrophages and can be upregulated upon activation with INF-$\gamma$, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4 [28]. PD-L1 and PD-L2 possess both costimulatory and coinhibitory actions on T cells. However, so far only the receptor initiating inhibitory functions, PD-1 (CD279), has been identified. PD-1 is expressed on activated T-cells, B-cells and monocytes and at a low level in natural killer (NK) cells [29]. PD-1 is upregulated after TCR or BCR engagement on naïve lymphocytes and persistent antigen stimulation maintains high PD-1 expression [30].

A major role of PD-1:PD-L interactions is to limit the activity of T-cells in peripheral tissues at the time of an inflammatory response to infection and to limit autoimmunity [31]. This regulation of peripheral tolerance by PD-1 was demonstrated in PD-1 deficient mice, which develop autoimmune diseases [32]. The PD-1:PD-L interaction inhibits T-lymphocyte proliferation, survival and effector functions (cytotoxicity, cytokine release), induces apoptosis of tumor-specific T cells, promotes the differentiation of $CD4^+$ T-cells into $Foxp3^+$ $T_{reg}$-cells, as well as the resistance of tumor cells to CTL attack [33].
In addition, the same PD-1:PD-L interaction is responsible for the functional impairment of antigen-specific $CD8^+$ T-cell responses during malignant transformation and chronic viral infections [34-36].

*Group III B7 molecules: B7-H3, B7x and B7-H6*

B7-H3 was identified soon after ICOS. Like other B7 family members, mouse B7-H3 mRNA is broadly expressed but protein expression is restricted to myeloid DC [37] where it is upregulated by lipopolysaccharides [38]. However, protein expression can also be induced in T-cells, NK cells and APC. This broad expression pattern suggests more diverse immunological and probably non-immunological functions of B7-H3, especially in peripheral tissues. Because the receptor for mouse and human B7-H3 has not yet been identified, functional analyses are currently difficult to perform, and the role of B7-H3 in T-cell regulation has still to be defined. Studies with *B7-H3*$^{-/-}$ mice, or mice treated with a B7-H3-blocking antibody exhibited enhanced experimental autoimmune encephalomyelitis (EAE), supporting an inhibitory function for B7-H3 [39]. In addition, experimental evidence implies that B7-H3 is involved in the regulation of cell growth and differentiation of non-hematopoietic tissues [40].

B7x mRNA expression occurs in peripheral tissues and in most activated hematopoietic and stromal cells, but protein expression is absent in most somatic tissues and only detected in epithelial cells of kidney, lung and pancreas. Like B7-H3, B7x engages a yet unidentified receptor on activated T-cells. B and T-lymphocyte attenuator (BTLA) was proposed as possible interaction partner of B7x, but subsequent investigations failed to confirm this interaction. However, functional studies show that B7x potently inhibits T-cell proliferation and IL-2 production and renders tumor cells refractory to apoptosis [41, 42].

B7-H6 is a PD-L1/B7-H3 homologue that specifically binds the CTLA-4-homologous NK-effector molecule NKp30. Unlike other B7 family members, B7-H6 is not expressed in any normal tissue even after activation, but is expressed in a variety of primary tumors and cell lines [43].

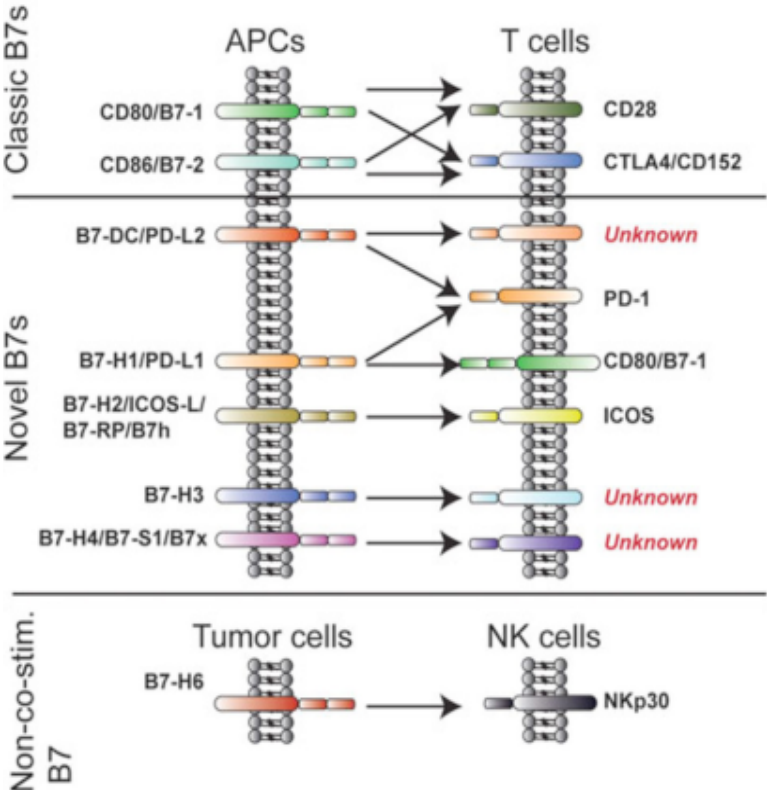



**Figure 2. Expression of selected antigens expressed on the surface of APC and their co-stimulatory or co-inhibitory ligands on the surface of T cells.** *Modified from Podojil and Miller, 2013. [44]*

### 1.1.3 Butyrophilin and Skint protein family

Structurally closely related to the B7 proteins is the butyrophilin (BTN) protein family. Murine BTN, in addition, display about 30% sequence identity with the *B7S3/SKINT* gene family, which mediate γδ T-cell differentiation [45]. Same as the B7 proteins, BTN and SKINT proteins are type 1 transmembrane glycoproteins belonging to the immunoglobulin (Ig) superfamily. BTN and BTN-like (BTNL) proteins are characterized by the presence of extracellular Ig-like domains (IgV and/or IgC), followed by a transmembrane domain and for most of the family members, a cytoplasmic B30.2 domain [46]. Similar to B7 proteins, BTN and SKINT proteins have been described with potential importance in cancer immune surveillance and immune modulation. The most extensively studied gene is the BTN family funding member *BTN1A1*. *BTN1A1* expression has been reported to be critical in the secretion of milk lipid droplets during lactation, a function that has been attributed to its cytoplasmic B30.2 domain [47, 48]. In addition, for some BTN and BTNL an involvement in T-cell regulation has been shown [49-52].

At present, little is known about the identity of the putative inhibitory receptor for the BTN proteins on T-cells. The receptor is unlikely to be any of the known inhibitory receptors on T-cells, namely CTLA-4, PD-1 or BTLA, all attempts to establish binding of Btn-Fc and Btnl-Fc to known receptors has been unsuccessful. So far, only human BTN2A1 has been shown to bind to DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), the C-type lectin molecule, a know entry receptor of the HIV virus in DC [53]. Notably, in many cases, binding was observed to activated but not unactivated T-cells, suggesting that BTN and BTNL proteins might engage T-cells that have already been activated, similar to the activity of CTLA-4 [54].

### 1.1.4 Evolution of the butyrophilin-locus

To date, 13 genes have been identified as *BTN* and *BTNL* genes in humans. The seven human *BTN* genes are all clustered on chromosome 6, in the MHC class I region, and are grouped into three classes that form phylogenetically associated groups: the single copy *BTN1 (BTN1A1)* gene and the *BTN2* and *BTN3* genes, which have undergone tandem duplication resulting in three copies of each type of gene, namely *BTN2A1*, *BTN2A2* and *BTN2A3*; and *BTN3A1*, *BTN3A2* and *BTN3A3*. In the mouse genome there are only two single gene copies, *Btnl1a1* and *Btn2a2*, which are orthologs of the human *BTN1A1* and *BTN2A2* genes, respectively. No ortholog for the human *BTN3* genes exists in mice. In addition to the *BTN* genes, the human genome contains a separate family of four *BTNL* genes: *BTNL2, BTNL3, BTNL8, BTNL9*, and the *BTN*-similar genes *erythroblast membrane-associated protein (ERMAP)* and *myelin oligodendrocyte glycoprotein* (*MOG)* [46]. he *BTNL* genes share considerable homology to the *BTN*-family members and like the *BTN* have similarly diverged significantly across species. *BTNL2*, the best-characterized family member, is clustered with the *BTN* genes on chromosome 6, whereas the much less explored family members *BTNL3*, *BTNL8* and *BTNL9* are localized on chromosome 5. In the mouse genome nine *Btn*-similar genes are described. Four of them, *Btnl2*, *Btnl9*, *Ermap* and *Mog,* are orthologs to human *BTNL* genes. The other five genes, *Btnl1*, *Btnl4*, *Btnl5*, *Btnl6* and *Btnl7,* have only been described in mouse. Note that *Btnl5* and *Btnl7* are predicted to be pseudogenes. The two *Btn* genes, *Btn1a1* and *Btn2a2* are localized on chromosome 13, whereas six *Btnl* genes are located in the MHC class II locus on chromosome 17, and *Btnl9* is found on chromosome 11. In addition a butyrophilin related *(BUR1)* pseudogene was found in human and mouse. However, no expression data regarding transcripts and protein exist for this group [55].

As illustrated in Figure 2, not all 14 BTN groups are conserved between species. Some groups have been duplicated in certain species, and other lost in other linages. In addition, some proteins have lost one of the three domains

(IgV, IgC and B30.2). All these events led to different BTN repertoires between species.

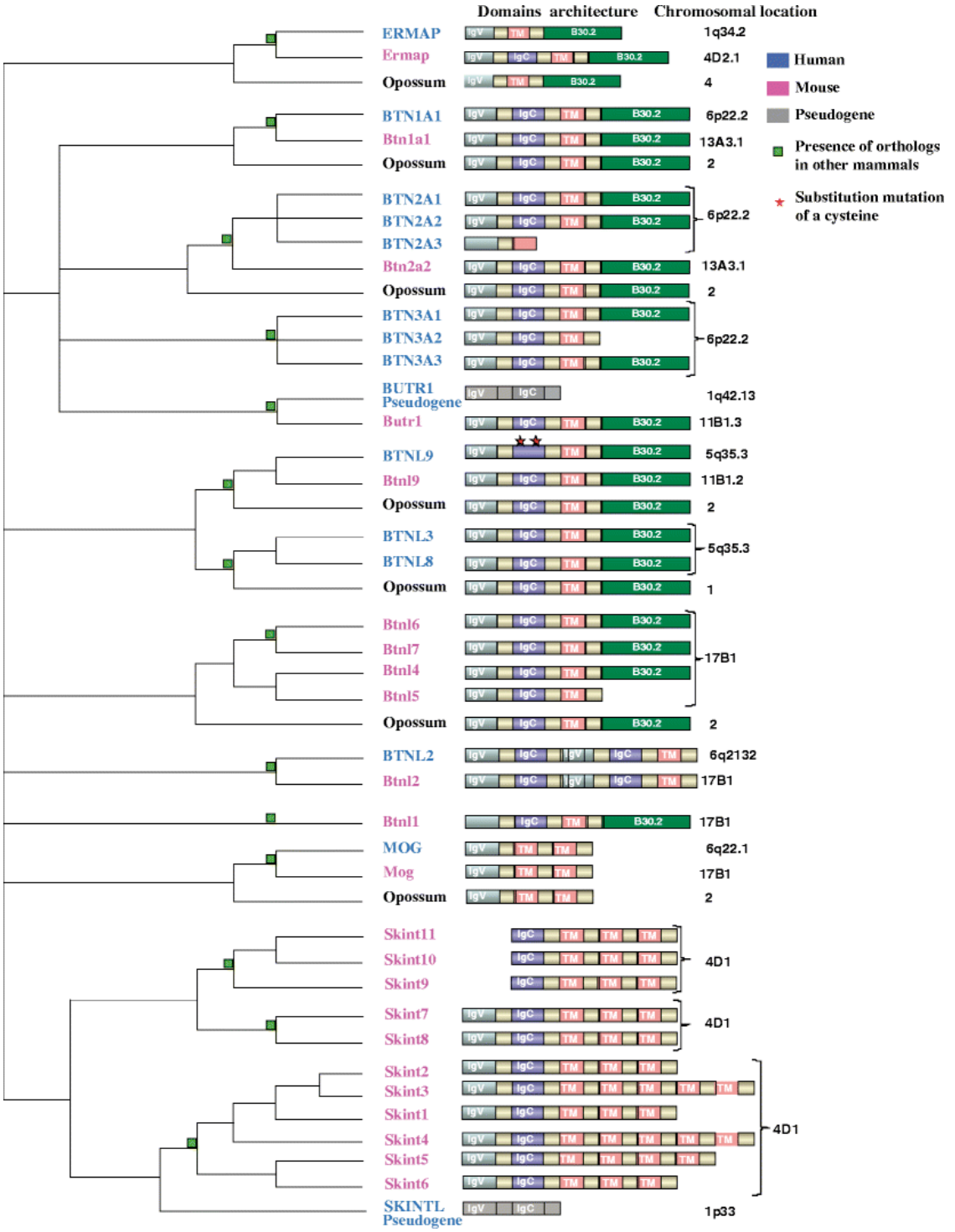



**Figure 3. Phylogenetic relationships between members of the butyrophilin family in mammals.** *BTN*, butyrophilin; *BTNL*, butryophilin-like; *BUTR1*, butyrophilin related 1; *MOG*, myelin oligodenrocyte glycoprotein; *ERMAP*, erythroid membrane-associated protein; *Skint*, selection and upkeep of intraepithelial T-cells. *Adapted from Afrache et al., 2012* [55].

### 1.1.5 Butyrophilins and immune function

The *BTN/BTNL* gene family exhibits several criteria of putative immune regulators. They are conserved in mice and humans, they share strong structural homologies with the B7 family, and several members are encoded within the MHC-locus. Indeed, like the B7 protein family, several murine and human BTN and BTNL family members have been shown to be immunologically active by controlling T-cell response [56]. However, while the B7 family of ligands and their receptors can regulate T-cell response either through their positive (e.g. B7.1, B7.2, ICOS-L) or negative (e.g. PD-L1, PD-L2, B7-H3, B7x) co-stimulatory molecules, BTNs so far almost exclusively have been found to act through co-inhibition [49, 50, 57, 58].

The first family member described to have co-stimulatory immune-function on the basis of B7 proteins, was murine BTNL2. Two groups demonstrated independently of each other in functional assays, that soluble BTNL2–FC fusion proteins inhibit proliferation and cytokine secretion of peripheral $CD4^+$ T-cells from the spleen, mesenteric lymph node, and Peyer's patch *in vitro*. Furthermore, *Btnl2* is overexpressed in the inflamed intestine of the *Mdr1a* knockout mouse model of spontaneous colitis, suggesting a role for *Btnl2* as a negative co-stimulatory molecule with implications for inflammatory disease and mucosal immunity in mice. However BTNL2 function on B-cells is unknown, it does not influence proliferation of B-cells that undergo activation through either LPS or the combination of anti-IgM and anti-CD40 [49].

Based on an analogous experimental design as for BTNL2, murine BTN1A1-FC and BTN2A2-FC fusion proteins were also shown to inhibit $CD4^+$ and $CD8^+$ T-cell activation *in vitro* [54], which could be reversed by an excess of co-stimulatory anti-CD8 antibodies. This mimics the actions of B7.1 and B7.2 molecules, suggesting that BTN work in a similar way.

Inhibitory effects on T-cells have also been reported for another murine BTNL molecule, BTNL1. Activated T-cells in the presence of recombinant *Btnl1* have been shown to inhibit $CD8^+$ T-cell proliferation by arresting cell-cycle progression [51]. Recently, BTNL1 has been found to regulate interactions with

intraepithelial γδ T-lymphocytes (IEL) [52] in the murine small intestine by suppressing proinflammatory mediators of the NFκB pathway, such as IL-6 and IL-15, and chemokines, such as chemokine CXC ligand 1 (CXCL1) and C-C motif ligand 4 (CCL4). In humans, the first BTN molecules found to engage receptors on T-cells and to inhibit T-cell activation were BTN1A1 and BTN2A2 *in vitro* [54].

Inhibitory actions on T-cells also have been reported for BTNL2. In addition, polymorphisms in *BTNL2* have been linked to a growing number of inflammatory diseases, all of them are characterized by an inappropriate T-cell activation e.g. sarcoidosis, ulcerative colitis, rheumatoid arthritis, spontaneous inclusion body myositis, systemic lupus erythematosus, type I diabetes, tuberculoid leprosy, and antigen-specific IgE responsiveness [59-65].

A very complex role in immune-regulation has been proposed for BTN3. It seems to be specific to cell-type and isoforms. Peripheral T-cell cultures, stimulated by CD3, show a reduction in proliferation and cytokine secretion after addition of an activating anti-BTN3A3 antibody [66]. In line, T-cells that interact with BTN3A3 on the surface of artificial APC show less expansion and produce less Th1-associated cytokines [67]. In contrary, BTN3A2 engagement enhanced TCR-induced signalling, cytokine production and proliferation of $CD4^+$ T-cells *in vitro* [68]. At last, another study reported that application of BTN3A1 antibody promotes a strong stimulation of phosphoagonists (PAg) activated Vγ9Vδ2 T-cells [69]. This induction could be inhibited by removing the B30.2 intracellular domain of BTN3A1, suggesting a direct role of the B30.2 domain in Vγ9Vδ2 stimulation [70].

### 1.1.6 B7 proteins and cancer

The immune system and cancer are interrelated at a very fundamental level. Both the innate and adaptive immune systems play a role in the prevention or promotion of tumorigenesis [71].

Cancer cells express tumor-specific aberrant antigens that differentiate them from normal cells, and must therefore evade immune surveillance to survive, either by inducing immunosupression or by deriving survival signals from tumor-infiltrating immune cells. Members of the B7 superfamily are involved in these processes, since the level of activation of the anti-tumor immune response depends on the balance between co-stimulatory and co-inhibitory signals [11]. In contrast to B7-1 and B7-2, whose expression is limited to lymphoid organs, many B7-H and BTN molecules can be expressed in non-lymphoid organs as well as on tumors cells in various cancers, where they are thought to contribute to tumor immune evasion [5].

Many of the B7-H family members are exploited by tumor cells to escape and suppress host immunity since the co-stimulatory pathways present elegant strategies to modulate T-cell response in autoimmune diseases and cancer. In addition, some co-inhibitory molecules are expressed on immune cell populations and may contribute to the escape of tumors to T-cell response by forming a shield for them. There expression on tumor cells provides a basis for an interaction between tumor cells and the host immune system [72].

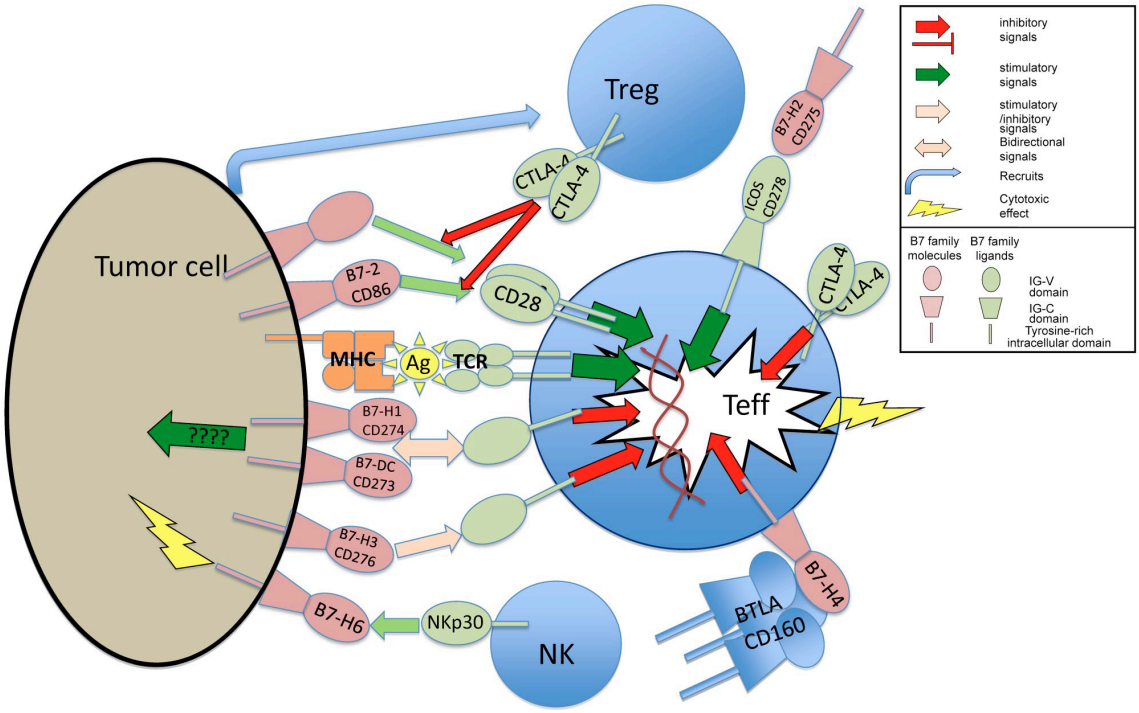



**Figure 5. Expression of B7 family members and their receptors have complex interactions with the tumor immune environment.** Treg indicates T-regulatory cells; Teff, T-effector cells; ????, limited evidence base for mechanism; Ag, antigen presented in MHC complex; MHC, major histocompatibility complex; and TCR, T-cell receptor complex. *From Greaves and Gribben, 2013* [8].

### 1.1.7 B7 proteins in hematological malignancies

*B7-1/B7-2:CTLA-4*

In contrast to solid tumors, B7-1 and B7-2 are expressed innately in many hematologic malignancies. After in vitro activation, follicular lymphoma (FL) cells upregulate B7-1 and B7-2 and, thereby, increase APC activity and augment primed T-cell response. In addition, high level of B7-1 and B7-2 are expressed on malignant Hodgkin Reed Sternberg (HRS) cells of classic Hodgkin lymphoma (CHL). However, expression of B7-1 and B7-2 is low in CLL and ALL [8]. Moreover, Ipilimumab has recently been approved by the Food and Drug Administration (FDA) as the first B7 pathway-targeting agent, anti-CTLA-4 monoclonal antibody for the treatment of metastatic melanoma [73].

*PD-L1/PD-L2:PD-1*

The PD-L1/PD-L2:PD-1 axis has been shown to contribute to failed antitumor immunity and upregulation of these molecules is associated with a poorer outcome in many hematologic malignancies including CLL and FL [74]. Long-lived residual leukemia cells isolated after treatment of murine ALL, were found to upregulate PD-L1, suggesting an involvement in leukemia persistence and relapse. PD-L1 is expressed at high level on a variety of hematopoietic malignancies such as acute myeloid leukemia, Hodgkin lymphoma, and myelodysplastic syndromes [75-77] Different PD-L1 antibodies (BMS-936558, Bristol-Myers Squibb; BMS-936559, Bristol-Myers Squibb; MPDL3280A) have been used in clinical trials blocking the PD-L1:PD-1 pathway on several types of solid cancer as e.g. NSCLC, lung, renal, melanoma, colon and castration resistant prostate cancer. In addition, promising results come from a phase I study of the anti-PD monoclonal antibody in advanced hematologic malignancies. Similar as PD-L1, high levels of PD-L2 expression have been described in cells of hematologic diseases, as acute myeloid leukemia and mantle cell lymphoma.

*ICOS-L:ICOS*

ICOS-L is widely heterogeneous expressed in myeloma and ALL. The ICOS-L:ICOS axis is thought to play an indirect role in enhancement of tumor immunity. ICOS is inducible on NK-cells showing cytotoxicity against ICOS-L-transfected murine leukemia cells and an optimal immunostimulatory therapy using CTLA-4 monoclonal antibody is thought to depend on an intact ICOS/ICOS-L pathway [78].

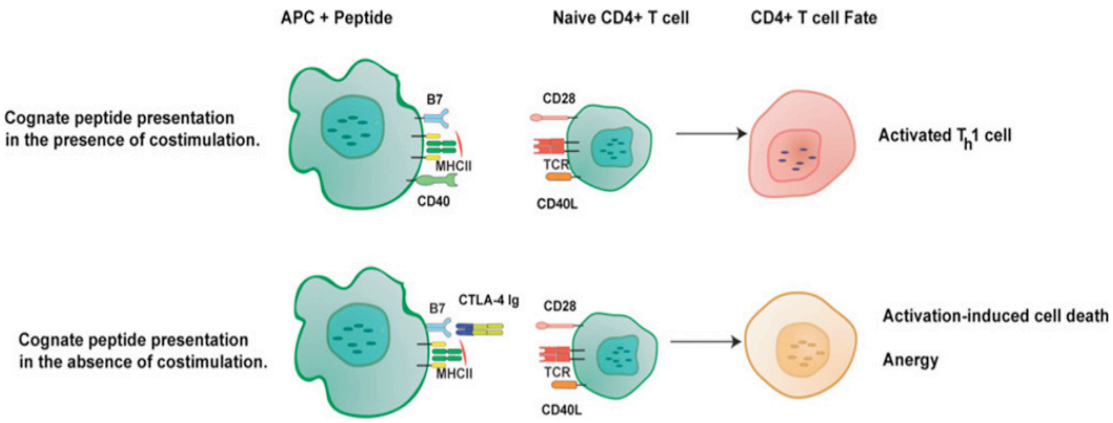



**Figure 4. T-cell activation in the presence of CTLA4-Ig.** *From Podojil and Miller, 2013.* [44]

## 1.2 Hematopoietic malignancies

Hematologic malignancies comprise a diverse group of disorders that affect the blood, bone marrow, and lymphatic system. As the three are intimately connected through the immune system, a disease affecting one of them will often affect the others as well.

In 2012, hematologic malignancies represented the fifth most commonly diagnosed cancers worldwide and the second leading cause of cancer death **http://globocan.iarc.fr**. In contrast to solid cancers, chromosomal translocations are a main cause of these diseases [79]. Hematological malignancies may derive from either of the two major blood cell lineages: myeloid and lymphoid cell lines.

Acute and chronic myelogenous leukemia, myelodysplastic syndrome and myeloproliferative disease are myeloid in origin and develop in granulocytes, erythrocytes, thrombocytes, macrophages and mast cells. In contrast, the lymphoid cell line produces B, T, NK and plasma cells and rearrangements in these cells lead to lymphoma, lymphocytic leukemia (acute and chronic) and multiple myeloma. However, hematological neoplasms have been historically most commonly divided by whether the malignancy is mainly located in the blood (leukemia) or in lymph nodes (lymphomas).

### 1.2.1 Leukemia

Leukemia (gr. λευκος-white and αιμα-blood) is a malignant disease that starts in blood-forming tissues such as the bone marrow.

The two main types of leukemia are lymphocytic leukemia, which involves an increase of white blood cells called lymphocytes; and myelogenous leukemia (also known as myeloid or myelocytic leukemia), which involves an increase in the number of granulocytes. In addition, different types of leukemia can be distinguished according to the clinical course of the disease in acute and chronic and depending on the maturation stage and origin of the cells. Acute leukemias have a rapid progression with a deadly outcome in weeks to month if untreated, whereas chronic leukemias have a less rapid clinical course (years) and predominantly occur in adults. Acute leukemias are thought to depend mainly on excessive proliferative signals, whereas the defect in cells of the chronic leukemia type mainly lies in apoptotic pathways.

In this line the clinical courses of both types vary, with rapid proliferating tumor cells (acute), opposed to slowly accumulating tumor cells (chronic) [80]. Therefore, the four main forms of leukemia that can be distinguished are: ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia;

**Table 2. The four main leukemia category**

| Cell type | Acute | Chronic |
|---|---|---|
| Lymphoblastic or Lymphocytic Leukemia | Acute Lymphoblastic Leukemia (ALL) | Chronic Lymphoblastic Leukemia (CLL) |
| Myeloid or Myelogenous Leukemia | Acute Myeloid Leukemia (AML) | Chronic Myeloid Leukemia (CML) |

The pathobiology of leukemia is not clear but in general it is thought to be a somatic genetic disorder of hematopoiesis in which one immune cell changes genetically and gives rise to a cell population with indefinite self-renewal

capacity, abnormal proliferation and differentiation, and a growth advantage over normal hematopoiesis. The exact mechanisms of tumorigenesis are unknown but it is thought to be a multistep process [81]. Each step leads ultimately to a general loss of responsiveness to signals that promote growth arrest, differentiation, or cell death. Common genetic alterations in tumors and leukemias are chromosomal translocations leading to the fusion of two unrelated genes and possible expression of a novel transforming fusion protein or deregulation of gene expression [82, 83].

### 1.2.2 Acute lymphoblastic leukemia (ALL)

Acute lymphoblastic leukemia (ALL) encompasses a group of malignant diseases of the bone marrow in which early B linage and T linage lymphoid precursors continuously multiply, replace the normal hematopoietic cell population and infiltrate other organs.

With representing nearly one third of all pediatric cancers, ALL is the most common malignancy diagnosed in children. Ninety percent of these childhood ALL cases involve the B cell lineage. There is a slight male predominance in all age groups and a significant excess incidence among white children. The five-years event-free survival rates for ALL now range between 76% and 86% in children receiving protocol-based therapy in developed countries [84].

The cause of most ALL is not known, however a variety of genetic and environmental factors have been related to ALL. It occurs with increased frequency in patients with Down syndrome, Fanconi anemia, Bloom syndrome, neurofibromatosis type 1, severe combined immunodeficiency, and ataxia-telangiectasia. In addition, exposure *in utero* to ionizing radiation, pesticides and solvents has also been related to an increased risk for childhood leukemia [85]. Common features of leukemic cells are an increased resistance to cell death and growth inhibitory signals, as well as and augmented self-renewal capability and proliferative capacity.

### 1.2.3 Pathophysiology of acute lymphoblastic leukemia

Normal lymphoid cell populations undergo several clonal rearrangements of their IG or T-cell receptor (TCR) genes.

Cells that successfully complete these genetic changes undergo a process of proliferation that results in the production of normal B and T cell populations. In ALL cells the normal lymphopoietic differentiation is disrupted, resulting in the generation of an excess of immature, non-functional lymphocytes, referred to as leukemic blasts. Uncontrolled clonal expansion of these transformed cells in the bone marrow perturbs normal hematopoieses, hindering production of functional blood cells and resulting in bone marrow failure.

Furthermore, this is accompanied by egress of the leukemic blasts from the bone marrow into the peripheral blood, frequently resulting in a potentially life-threatening high white blood cell count (WBC). Concomitantly, these blasts can also infiltrate extramedullary tissues such as, e.g., liver, spleen, lymph nodes and the central nervous system (CNS).

In ALL, this pattern emerges quickly; at first, patients suffering from ALL display diffuse symptoms of general unwellness, decreased fitness, bruising, anemia, fever and high susceptibility to infections, which all can be directly linked to the disrupted blood cell generation in the bone marrow. In addition, infiltration and accumulation of the blasts in extramedullary organs results in painful enlargements, which may compromise normal organ function; hepatomegaly and splenomegaly are often present at diagnosis [86].

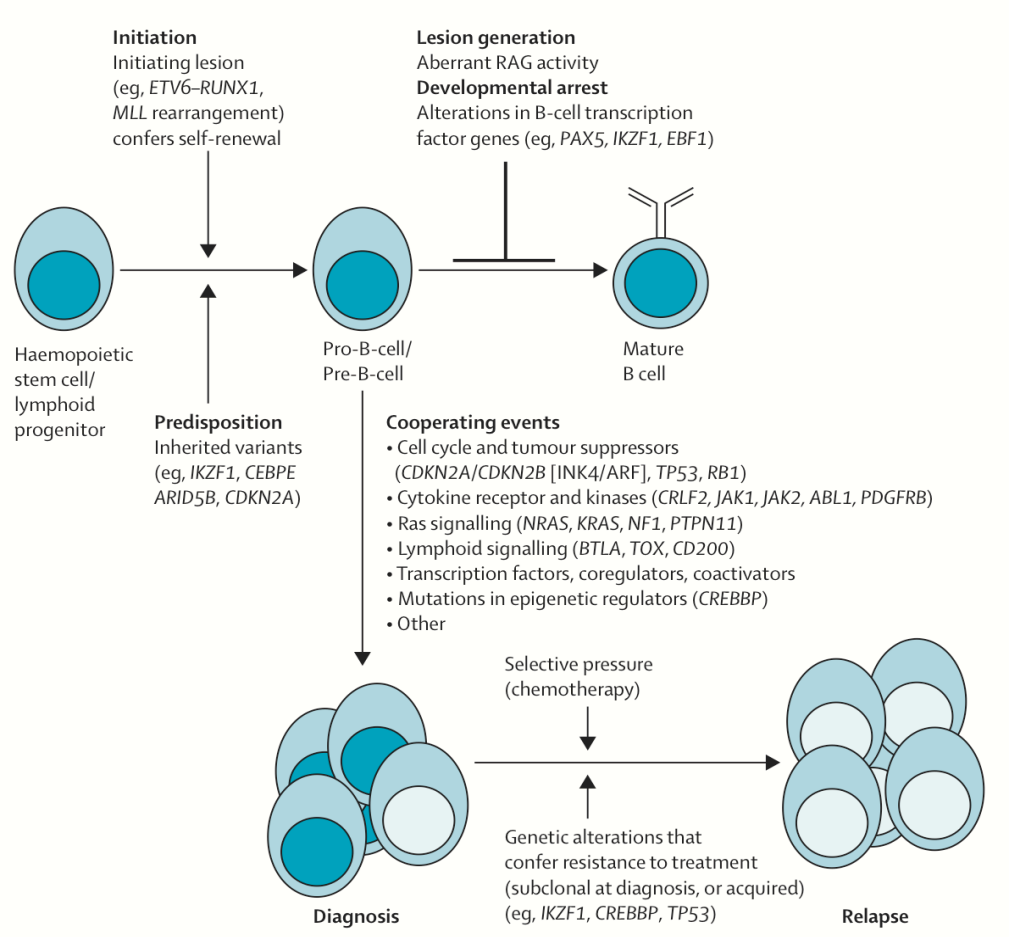



**Figure 6. Genetic pathogenesis of B-ALL at diagnosis and relapse.** *From Mullighan, 2013.* [87]

### 1.2.4 Classification of acute lymphoblastic leukemia

ALL is a disease of highly heterogeneous presentation; as a result, ALL can be subdivided according to several criteria.

*Age:* The most commonly used system is the age at presentation, which defines the disease as infant (<12 months), pediatric (1–18 years) and adult ALL (>18 years). However, there is a stage concerning late adolescent and young adult ALL patients (15–25 years), where classification and treatment often occurs according to the patient referral to either a pediatric or an adult oncologist; some clinical studies define childhood ALL and concomitant eligibility for a trial until age of 25.

*Immunophenotype:* According to the hematopoietic cell linage of the blasts, the two main categories are T-cell precursor (TCP) and B cell precursor (BCP) ALL; characterized by T-cell linage (CD3) and B linage CD markers (CD19, CyCD79), respectively. Much rarer is a biphenotypic acute leukemia (BAL), where lymphoid and myeloid or B- and T-cell markers are co-expressed.

*Karyotype/cytogenetic subtype:* In contrast to solid tumors, genomic rearrangements are a hallmark of leukemia. Structural abnormalities are recurring inter- and intra-chromosomal rearrangements between specific loci, resulting in derivative chromosomes, frequently coding for fusion oncogenes. Other structural lesions apart from rearrangements can be partial loss of specific chromosomes, for instance deletion of the p or q arm [del(p)/del(q)]. Both numerical and structural genetic lesions represent the cytogenetic subtype of the disease and define clinical entities with specific underlying pathobiologies; some are predictors of outcome, and as such, the cytogenetic phenotype is used for therapy stratification of ALL patients.

*Morphology:* Historically, ALL blasts have been categorized according to morphologic parameters, proposed by the French-American-British (FAB) - classification system. Cell size, nucleus to cytoplasm (N/C) ratio, appearance of nucleoli and the shape of the nuclear membrane are assessed and assigned a specific value; the final sum determining the cytomorphological classification of

the blasts.

**Table 3. FAB classification of ALLs**

| Type | Morphology |
|---|---|
| L1 - ALL | Small, uniform blasts with high N/C ratio, undefined nucleolus and smooth nuclear membrane. |
| L2 - ALL | Large, varied blasts with varying N/C ratio, distinct multiple nucleoli and irregular nuclear shape. |
| L3 - ALL | Large, varied blasts with low N/C ratio, vacuolated cytoplasm as well as distinct nucleoli. |

However, the recent WHO proposed classification of ALL recommends that the FAB classification be abandoned, since the morphological classification has no clinical or prognostic relevance. It instead advocated the use of the immunophenotypic classification mentioned below.

1. Acute lymphoblastic leukemia; Synonyms: Former Fab L1/L2
    i. Precursor B acute lymphoblastic leukemia. Cytogenetic subtypes:
        - t(12;21)(p12,q22) TEL/AML-1
        - t(1;19)(q23;p13) PBX/E2A
        - t(9;22)(q34;q11) ABL/BCR
        - T(V,11)(V;q23) V/MLL
    ii. Precursor T acute lymphoblastic leukemia/lymphoma
2. Burkitt's leukemia; Synonyms: Former FAB L3
3. Biphenotypic acute leukemia

### 1.2.5 Genetics of acute lymphoblastic leukemia

ALL is a highly heterogeneous disease that includes many subtypes defined by recurring chromosomal alterations that are important events in leukemogenesis and are widely used in diagnosis therapy and prognosis.

One of the more common chromosomal abnormalities in ALL include the t(12;21)(p12;q22) translocation which lead to the TEL-AML1 fusion gene, which can be found in 25% of cases of pre-B ALL. The presence of this translocation carries a more favorable prognosis.

Moreover, the *BCR-ABL* t(9;22)(q34;q11) translocation is found in only about 3% to 5% of cases of childhood ALL. The presence of this translocation is associated with a high WBC count at diagnosis and a poor response to therapy [87].

Another major cytogenetic subgroup is marked by rearrangements involving the gene locus 11q23, which encodes the *MLL* (*Mixed Lineage Leukemia*) gene, occurring in approximately 2-8% of pre-B ALL cases. However, rearrangements of the MLL gene are found in over 70% of ALL cases in infants. *MLL* normally functions as a transcription regulator of the *HOX* genes and is essential for normal mammalian development and hematopoiesis. Unfortunately, young children with this genetic abnormality have a very poor prognosis and a survival of less than 20% despite intensive therapy. Children, with *MLL* gene rearrangements, less than 1 year of age at diagnosis were found to have better prognoses than those of infants with the same translocation, but far worse than age-matched patients without rearrangements of the *MLL* gene [88].

**Table 4. Correlation of prognosis with genomic rearrangements in ALL**

| Cytogenetic change | Risk category |
|---|---|
| Philadelphia chromosome | Poor prognosis |
| t(4;11)(q21;q23) *MLL-AF4* | Poor prognosis |
| t(8;14)(q24.1;q32) *IGH@-MYC* | Poor prognosis |
| Complex karyotype (more than four abnormalities) | Poor prognosis |
| Low hypodiploidy or near triploidy | Poor prognosis |
| High hyperdiploidy (specifically, trisomy 4, 10, 17) | Good prognosis |
| del(9p) | Good prognosis |

### 1.2.6 Treatment of acute lymphoblastic leukemia

Treatment for ALL can include chemotherapy, steroids, radiation therapy and growth factors.

Classical protocols used to treat ALL are made up of distinct phases comprising multiple chemotherapeutic agents, with a total duration of two years. Treatment begins with a three- or four-drug induction phase, with the aim of killing all leukemic cells within the first 4–5 weeks. Remission induction regimens usually include a synthetic glucocorticoid (prednisone or dexamethasone), vincristine, asparaginase and daunorbicin. This is followed by phases of consolidation/intensification, re-induction and then maintenance with a total of up to 11 different agents, which aims to eliminate residual leukemic blasts and effect cure. In particular, the introduction of an intensive re-induction phase has significantly improved survival rates.

Despite these figures, nearly 20% of children with ALL will relapse, and survival after relapse is poor, particularly in high-risk patients [89].

## 1.3 Glucocorticoids

Glucocorticoids (GC) are a class of steroid hormones secreted by the adrenal glands that exert a wide range of anti-inflammatory and immune-suppressive activities.

Therefore, numerous high-affinity synthetic GC such as prednisone (Pred) and dexamethasone (Dex) are commonly used in the treatment of inflammatory and autoimmune diseases. However, prolonged use of these compounds is complicated by numerous deleterious side effects such as hypertension, osteoporosis, psychosis Cushing's syndrome and leucopenia [90].

In addition, the ability of GCs to induce cell cycle arrest and apoptosis in lymphoid cells has led to their inclusion in chemotherapy protocols for many hematological malignancies [91-93]. However, development of GC resistance still is one of the main problems in the treatment of lymphoid malignancies.

### 1.3.1 Glucocorticoid receptor and function

The effects of GC are mediated by the ubiquitously expressed glucocorticoid receptor (GR) also known as NR3C1 (nuclear receptor subfamily 3, group C, member 1) a member of the type I nuclear hormone receptor super family of ligand-activated transcription factors.

The human *GR* gene encodes nine exons and is located on chromosome 5q31.3. Alternative splicing of exon nine of the *GR* gene generates two highly homologous receptor isoforms (α and ß). They are identical through amino acid 727 but then diverge, with GRα having an additional 50 amino acids and GRß having an additional, non-homologous 15 amino acids.

Full-length GRα is the predominantly expressed form in human tissues. As all members of the nuclear hormone receptor super family, both GR isoforms consists of three distinct structural and functional domains. The N-terminal region domain (NTD) contains a ligand independent transactivation domain, termed activation function (AF)-1. The central DNA binding domain (DBD) consists of two highly conserved zinc finger motifs and is essential for binding to GC response element (GRE) sequences of regulated genes. The first zinc finger motif is necessary for binding to nuclear factor (NF)-κB and AP-1 transcription factor and for the transrepression function of the GR. The second zinc finger domain is involved in receptor dimerization and transactivation via GRE-binding in the promoter region of many target genes. The region between the two zinc fingers contains a nuclear export signal (NES). In addition, a hinge region adjacent to the DBD houses a nuclear localization sequence (NLS). The C-terminal region contains a ligand-binding domain (LBD), which plays a crucial role in the ligand binding and cofactor binding activity of the GR. The LBD also contains another weak transactivation domain, AF-2. The activity of AF-2 is ligand-dependent [94].

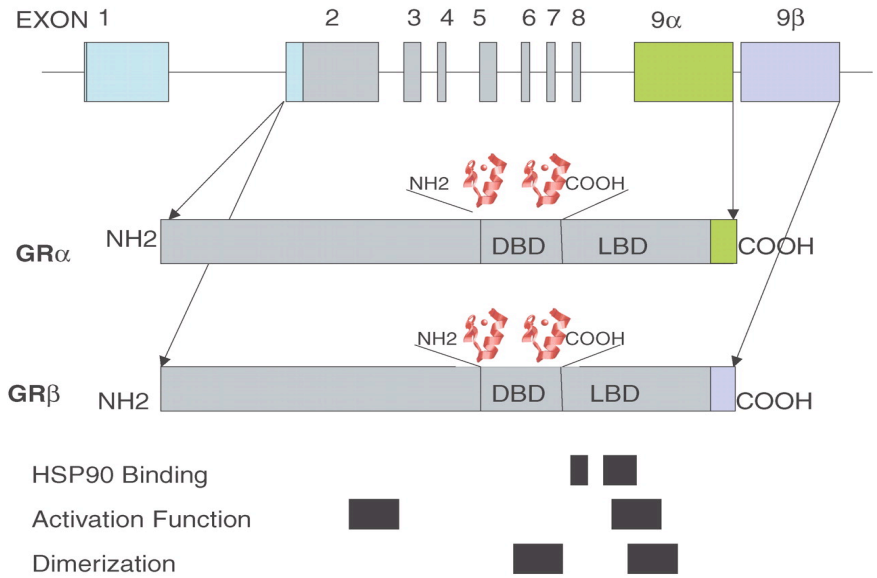



**Figure 7. Genomic and functional structure of the glucocorticoid receptor.** Exon regions are indicated by grey boxes, isoforms indicated by different colors *(McMaster and Ray, 2005).*

In the absence of its ligand, GR associates with a heat shock protein (HSP) complex in the cytoplasm. Upon ligand binding, the receptor undergoes a conformational change, dissociates from the complex and subsequently translocates to the nucleus where it activates or represses the transcription of GC-responsive genes [95] . The induction of genes by GR is mediated via GR interaction with conserved GREs, whereas gene repression occurs through negative GREs (nGREs), protein-protein interaction with other transcription factors, competition for co-activators and other mechanisms. After modulating the transcription of its responsive genes, GR dissociates from the ligand and slowly returns to the cytoplasm as a component of heterocomplexes with HSP [96].

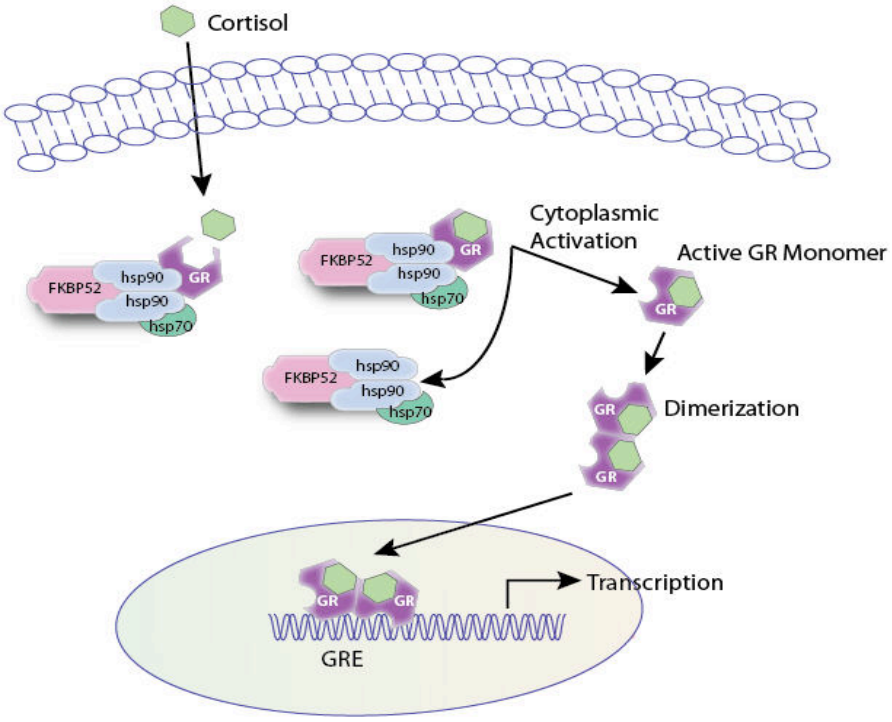



**Figure 8. Activation of GR.** GR, glucocorticoid receptor; GRE, glucocorticoid response element; *From Baschant and Tuckermann, 2010* [97].

Due to the broad distribution of GC and their cognate receptors, GC signaling controls a wide range of physiological actions on mammalian cells and entire organism. For example in the liver and adipose tissue, GC positively regulate metabolism through the stimulation of gluconogenesis and lipolysis, respectively [98].

In contrast, in the immune compartment, GCs act largely inhibitory, causing immune suppression and the inhibition of inflammation via repression of pro-inflammatory cytokines. Moreover, in a number of systems, including acute lymphoblastic lymphoma (ALL), GC induce apoptosis and cell cycle arrest. This broad bioavailability and diverse physiological effects have made synthetic GCs one of the most frequently prescribed drugs worldwide [99].

### 1.3.2 Glucocorticoid-induced apoptosis

The mechanism behind GC-induced apoptosis is not fully understood and seems to vary depending on cell type.

However, traditionally, GC-induced apoptosis is divided into three stages: 1) initiation stage, which involves GR activation and GR-mediated gene regulation; 2) decision stage, which engages the prosurvival and proapoptotic factors at the mitochondrial level; and 3) execution stage, which implicates caspases and endonuclease activation.

1) GC-induced apoptosis is initiated by, and strictly dependent upon, the interaction of GC with its receptor, the GR. A critical determinant to induce apoptosis via GR is its expression-level as evidenced by work in transgenic mice with increased and decreased GR expression [100, 101]. In addition, a phenomenon known as GR autoinduction, the ability of cells to up-regulate the GR in response to GC exposure, is required for GC-induced apoptosis [102, 103]. There is evidence that GC-induced apoptosis depends on initiation of transactivation but not transrepression by the GR. So does GC-induced apoptosis of lymphocytes not progress in the presence of actinomycin D and cycloheximidine, indicating a requirement for *de novo* transcription and translation in the execution of the apoptotic cascade [104]. Studies using thymocytes from genetically modified mice, expressing point mutations in GR to repress transactivation, and human acute lymphoblastic leukemia (ALL) cell lines with mutated GR further support this observation by showing a failure to undergo GC-induced apoptosis [105].

2) There are several indications that GCs can act on the extrinsic (extracellular inducers) pathway. In addition, there is considerable evidence that GC-induced apoptosis proceeds through the intrinsic (intracellular inducers) pathway. So exhibit thymocytes from caspase-9 deficient mice reduced sensitivity to GC-induced apoptosis [106]**.** In addition, it has been shown that Dex induces a loss of mitochondrial membrane potential in thymocytes and T-cell hybridoma cells [107].

3) The GC-induced apoptosis pathway culminates in the activation of a class of proteins known as caspases. Caspases are a family of proteases that cleave substrates at asparate residues [108], a signaling pathway referred to as the ‘caspase cascade’. Studies using a broad spectrum of caspase inhibitors found that GC-induced apoptosis requires caspase activation [109].

### 1.3.3 Regulation of glucocorticoid sensitive genes

It is widely accepted that GC-induced apoptosis results from alterations in gene expression. However, up to this date only a few genes have been shown to directly be involved in GC-induced apoptosis. Most notably, GC can activate cell death through induction of pro-apoptotic members of the BH3-only subgroup of the BCL-2 family, such as BIM, BID and BAD and/or repression of anti-apoptotic members, such as BCL-2, BCL-XL and MCL-1 [110].

The expression of *Bim* is induced by GC treatment in murine lymphoma cell lines, mouse primary thymocytes, human leukemic cell lines and human primary chronic lymphoblastic leukemia (CLL) and acute lymphoblastic leukemia (ALL) samples. Isolated thymocytes from *Bim* knock-out mice exhibit a significantly decreased sensitivity to GC-induced apoptosis [111] making BIM one of the key-players in GC-induced apoptosis. Since *Bim* does not harbor a GRE in its promoter-region it is assumed that its activation by GR is indirect. Recent studies found that the activity of the serine/threonine kinase GSK3 mediates GC-induced apoptosis by up-regulating *Bim* expression [112]. The induction and activation of *Bim* leads to activation of the downstream apoptotic mediators BAX and BAK. Upon activation, BAX and BAK mediate the destabilization of the mitochondrial membrane potential, a hallmark of the intrinsic mitochondrial apoptosis pathway [113].

In addition to *Bim*, GCs rapidly transactivate *glucocorticoid-induced leucine zipper* (*GILZ*) in several systems. *GILZ* presents three GREs in its promoter-region, therefore GC-induction of *GILZ* expression is direct and strong [114]. Isolated primary thymocytes of GILZ-deficient mice were resistant to TCR-induced apoptosis. However, they exhibited augmented GC-induced apoptosis due to reduced expression of the anti-apoptotic BCL-2 family member BCL-XL, as well as increased activation of caspases 9, 8 and 3 [115]. GILZ also mediated GC-induced cell cycle arrest through inhibition of the proliferative RAS and RAF oncogenes [116].

Another gene being found upregulated by GCs in murine lymphoma cell lines is the stress gene dexamethasone-induced gene 2 (Dig2). Interestingly, other than

BIM and GILZ, DIG2 overexpression reduced the sensitivity of these cells to GC-induced apoptosis, suggesting a pro-survival function for this gene [117]

T-cell death-associated gene (TDAG8) is rapidly induced by GCs in thymocytes. Thymocytes from TDAG8 knock-out mice exhibited increased activation of caspases 3, 8 and 9 following GC exposure [118]. Moreover, GC exposure represses the pro-survival oncogene c-MYC in human CEM cells [117].

### 1.3.4 Resistance to glucocorticoid therapy

A main problem of GC chemotherapy is the sudden emergence of GC-resistant clonal populations during GC therapy, GC resistance during relapse and the existence of inherently resistant malignancies.

Resistance can occur on the level of the entire organism, as in primary cortisol resistance, or affect the descendants of a particular cell clone, as in ALL. Patients with relapsed ALL exhibit a significantly increased resistance to GC therapy [119] GC resistance in these cancers is associated with a poor prognosis [120].

A large number of possible molecular mechanism for GC resistance can be envisaged acting either ‘upstream’, at the level of the GR, or ‘downstream’, at the level of the GC-regulated genes, in the GC-triggered signaling pathway. Therefore, a more comprehensive understanding of the factors governing GC resistance in hematomalignancies may improve the efficacy of GC therapy. Furthermore, leukemias of the myelogenous linage are often innately resistant to GC therapy [121].

# OBJECTIVES

The main objective of this thesis is to functionally characterize the human *BTNL* gene cluster and to and evaluate possible implications in disease.

The detailed objectives are:

- ✓ To investigate the *BTNL* gene cluster at human chromosome 5q35.3 in a population-genetic analysis (Results I).
- ✓ To identify functional consequences of the *BTNL8-BTNL3* deletion copy number variant (Results I).
- ✓ To elucidate biological functions of the characterized *BTNL* genes, which could provide an insight in human biology (Results II).

# RESULTS

This PhD thesis is based on the following original scientific communications:

I. **Aigner J**, Villatoro S, Rabionet R, Roquer J, Jiménez-Conde J, Martí E and Estivill X (2013). “A common 56-kilobase deletion in a primate-specific segmental duplication creates a novel butyrophilin-like protein”. *BMC genetics*. 14:61.

II. **Aigner J,** Martí E and Estivill X (2013). “Butyrophilin-9 (*BTNL9*), a novel glucocorticoid sensitive gene promotes resistance in *MLL-AF4* rearranged acute lymphoblastic leukemia (ALL)”. *Manuscript submitted.*

# RESULTS I:

## “A common 56-kilobase deletion in a primate-specific segmental duplication creates a novel butyrophilin-like protein”

**Aigner J**, Villatoro S, Rabionet R, Roquer J, Jiménez-Conde J, Martí E and Estivill X. A common 56-kilobase deletion in a primate-specific segmental duplication creates a novel butyrophilin-like protein. *BMC genetics*. 2013;14:61.

Aigner J, Villatoro S, Rabionet R, Roquer J, Jiménez-Conde J, Martí E and Estivill X. A common 56-kilobase deletion in a primate-specific segmental duplication creates a novel butyrophilin-like protein. Supplementary information. *BMC genetics*. 2013; 14:61.

# RESULTS II:

"Butyrophilin-9 (*BTNL9*), a novel glucocorticoid sensitive gene promotes resistance in *MLL-AF4* rearranged acute lymphoblastic leukemia (ALL)"

# Butyrophilin-9 *(BTNL9),* a novel glucocorticoid sensitive gene, promotes dexamethasone resistance in *MLL-AF4* rearranged acute lymphoblastic leukemia (ALL)

**Johanna Aigner[1,2,3,4], Jessica Nordlund[5], Eulalia Martí[1,2,3,4], Xavier Estivill[1,2,3,4,]**

[1]Genomics and Disease Group, Bioinformatics and Genomics Program, Centre for Genomic Regulation (CRG), 08003 Barcelona, Spain

[2]Universitat Pompeu Fabra (UPF), 08003 Barcelona, Spain

[3]Centro de Investigación Biomédica en Red en Epidemiología y Salud Pública (CIBERESP), 08003 Barcelona, Spain

[4]Hospital del Mar Medical Research Institute (IMIM), 08003 Barcelona, Spain

[5]Department of Medical Sciences, Molecular Medicine and Science for Life Laboratory, Uppsala University, 75185 Uppsala, Sweden

*Corresponding author

## Abstract

Resistance to glucocorticoids (GC) remains an enormous problem in the treatment of acute lymphoblastic leukemia (ALL), especially in ALL cases of mixed-lineage leukemia (*MLL)* gene rearrangements GC resistance is associated with a poor clinical outcome. Understanding the process that leads to GC resistance remains an important step to improve prognosis of this type of ALL. Here we report the identification of a novel GC-induced gene, *BTNL9,* which is upregulated following dexamethasone treatment, through a mechanism directly dependent on the glucocorticoid receptor.

High *BTNL9* expression-levels recently have been associated with high-risk of bad clinical outcome in ALL infants with chromosomal translocations t(4;11), involving *MLL and AF4*. Here we show, that *BTNL9* mediated GC-resistance in *MLL-AF4* rearranged ALL cell lines, and that downregulation of BTNL9 led to an increase in GC sensitization. In line, enforced *BTNL9* expression in GC-sensitive cell lines increased resistance to GC treatment. Moreover, we show that *BTNL9* was upregulated in response to a wide variety of other apoptosis-inducing drugs, including staurosporine, etoposide, and retinoic acid.

We conclude that *BTNL9* is as a novel pro-survival GC-sensitive gene with a general function in apoptosis and especially in GC-resistant *MLL* rearranged ALL. We propose that inhibiting *BTNL9* could improve the outcome in GC-resistant *MLL* rearranged ALL patients.

## Introduction

Acute lymphoblastic leukemia (ALL) is a neoplasm of immature lymphoid progenitors that can occur in the early B and T cell lineage, whereas the immature CD10-negative B linage precursor ALL (pre-B ALL) is the more common form [1]. Pre-B ALL is characterized by a high incidence of balanced chromosomal translocations involving the *mixed-lineage leukemia* (*MLL*, *ALL-1* or *trithorax homolog; HRX)* gene arising during embryonic development [2]. Rearrangement of the *MLL* gene on chromosome 11q23 occurs in ~80% of infants (>1 year of age) diagnosed with ALL and in ~85% of secondary leukemias, that arise in patients treated with epipodophyllotoxins and other DNA topoisomerase II inhibitors [3]. Up to date, over 60 *MLL*-translocation partners have been identified, and the *MLL-AF4* translocation counts for ~50% of all cases [4].

Same as the *MLL* gene, *AF4 (AFF1*, *AF4/FMR2* family, member 1) belongs to a transcriptional transactivating gene family [5]. The translocation usually fuses the N-terminal portion of the *MLL* gene to the C-terminal region of its translocation partner [6]. The *MLL* gene encodes a member of the trithorax protein family that positively regulates gene expression, including multiple genes of the *HOX* family. Characteristic for *MLL*-fusion proteins is the loss of the histone H3 lysine 4 (H3K4) methyltransferase (SET) domain, which leads to aberrant histone modifications and results in an altered chromatin remodeling [7]. This leads to a gene expression profile clearly distinguishable from *MLL* germline ALL, indicating that *MLL* rearranged ALL is a distinct biological entity that responds poorly to conventional ALL-directed therapy.

A great challenge today is to develop strategies that can overcome the drug resistant phenotype. For this purpose it is important to understand the underlying mechanisms of GC resistance and the signaling pathways regulating apoptosis induced by GCs.Patients diagnosed with *MLL* rearranged ALL have an especially poor outcome compared with children with other forms of ALL. This poor treatment outcome is mainly due to cellular drug resistance, in particular resistance to synthetic glucocorticoids (GC) like prednisolone and

dexamethasone [8]. GC are effectively used in the treatment of various hematopoietic malignancies due to their ability to induce apoptosis in these cancerous cells. The effects of GC are mediated through the glucocorticoid receptor (GR), which after ligand binding translocates from the cytosol to the nucleus where it affects expression of numerous genes through transactivation and transrepression [9].

The butyrophilin-like (BTNL) protein family is structurally closely related to the B7 family and is thought to play an important role in cancer immune surveillance and immune modulation [10]. Recently, we identified a 56 kilobase (kb) deletion copy number variant (CNV) that affects expression of *BTNL9* in lymphoblastoid cell lines (LCL) [11]. *BTNL9* has been found to be over-expressed in hematopoietic malignancies, including germinal center B cell like (GBC) type of diffuse large B cell lymphoma (DLBCL) [12] and follicular lymphoma (FL) [13]. In addition, *BTNL9* is differentially expressed in pre B-ALL after GC treatment [14]. However, up to this date, data on *BTNL9* expression on ALL are limited.

In this study, we confirm *BTNL9* as a GC sensitive gene that is upregulated after dexamethasone treatment in pre-B ALL cell lines. Moreover, we demonstrate that pre B-ALL cell lines expressing *BTNL9* are more resistant to GC induced apoptosis and that this phenotype can be reversed by downregulating endogenous *BTNL9* expression. In addition, over-expression of BTNL9 significantly reduced GC sensitivity in *MLL-AF4* rearranged ALL cell lines, making BTNL9 an interesting new target for therapy of this type of ALL.

## Results

### *BTNL9* is upregulated in cell lines resistant to GC-treatment

Five pre-B ALL cell lines were purchased from the German collection of microorganism and cell cultures (DSMZ), and MV4;11 and BCL-1 were available in the laboratory. All cell lines were genotyped for the *BTNL8-BTNL3* deletion CNV as described previously [11]. One cell line (MV4;11) was homozygous for the wild-type allele, three (BEL-1, MHH call2 and RSA4;11) were heterozygous, and three (NALM-6, REH and SEM) were homozygous for the deletion variant. mRNA was isolated and *BTNL9* expression levels were measured by quantitative real-time PCR (qPCR) in all seven pre-B ALL cell lines. Previously, it was shown by our group, that LCL harboring the *BTNL8_BTNL3-del* allele have a reduced expression of *BTNL9* [11].

In line with our previous findings in LCL, the cell line homozygous for the wild-type allele (*BTNL8-BTNL3* non-deletion allele), MV4;11, showed high *BTNL9* expression, MHH call2 showed a moderate expression level, while NALM-6 and REH almost did not express *BTNL9*. However, *BTNL9* mRNA level was unusually low in the cell lines BEL-1 and RSA4;11, and very high in SEM (Figure 1a). Next, apoptosis rate was measured in all cell lines after 24 h of dexamethasone treatment. The *MLL-AF4* rearranged cell lines, MV4;11 and SEM were GC resistant, while BEL-1 and RSA4;11 were GC sensitive (Figure 1 b). In addition, the *MLL* germline ALL cell lines MHH call2 and REH were GC resistant, and NALM-6 was moderately GC sensitive (Figure 1c).

Interestingly, cell lines expressing high-level *BTNL9* are poor-responding or resistant to GC-induced apoptosis (black bars), while cell lines expressing low-level *BTNL9* are sensitive to GC-induced apoptosis (grey bars). Note that REH does not express *BTNL9* even though it is highly resistant to GC-induced apoptosis (Figure 1a). However, this resistance is known to be mediated through a dysfunctional glucocorticoid receptor (GR) (Figure 1c).

## *BTNL9* is induced by dexamethasone and requires transcriptional activation mediated through the GR

*BTNL9* has previously been shown to be upregulated in pre-B ALL cells *in vivo* after treatment with the synthetic GC prednisolone [14, 15]. To confirm the induction of *BTNL9* by GC, mRNA was isolated and qPCR was performed on the pre-B ALL cell lines MHH call2, SEM and REH treated with 1 μM dexamethasone for 3, 6, 12, 24 and 48 h. In the cell lines MHH call2 and SEM, *BTNL9* mRNA level was elevated immediately after 3 h and reached its maximum after 6 h (Figure 2a). However, in the GC-resistant cell line REH, *BTNL9* expression was not induced by dexamethasone, indicating a requirement of a functional GR for the induction of *BTNL9* (Figure 2a). Similar results were obtained using prednisolone, a closely related GC (Supplementary Figure 1).

The rapid induction of *BTNL9* by dexamethasone suggested a primary transcriptional response. To test the role of GR-mediated transcription in the induction of *BTNL9* by dexamethasone, the pre-B ALL cell lines MHH call2 and SEM were cultured in the presence of 1 μM of dexamethasone and a 10-fold excess of the GR antagonist RU486, to block GR activation by dexamethasone. In the presence of RU486, induction of *BTNL9* mRNA by dexamethasone was completely inhibited, indicating a requirement of GR activation for the induction of *BTNL9* (Figure 2b).

## Down-regulation of *BTNL9* in *MLL-AF4* rearranged ALL cells mediates sensitivity to GC

Recently, high-level *BTNL9* was found to correlate with high-risk in infant *MLL-AF4* rearranged ALL *in vivo* [16]. In line with this finding, *BTNL9* expression was very low in the GC good-responding cell lines RSA4;11 and BEL-1 and very high in the GC poor-responding cell lines SEM and MV4;11, independent of the *BTNL8_BTNL3-del* allele (Figure 1a).

To check whether high *BTNL9* expression-level correlates with a higher GC resistance in these cells, endogenous *BTNL9* was down-regulated in the GC-

resistant *MLL-AF4* rearranged ALL and AML cell lines SEM and MV4;11 with the use of RNA interference. In addition *BTNL9* was downregulated in the poor GC responding *MLL* germline ALL cell line MHH call2. Western blot analysis was used to measure the efficiency of BTNL9 repression after 48 h of treatment with 1 μM dexamethasone. As shown in Figure 3a, protein expression of dexamethasone-induced BTNL9 was successfully decreased in *BTNL9* siRNA treated cells compared to cells transfected with control siRNA. Compared with control cells, the two *MLL-AF4* rearranged ALL and AML cell lines, SEM and MV4;11, expressing *BTNL9* siRNA, became significantly more sensitive to dexamethasone-induced apoptosis (Figure 3b). In addition, a moderate increase in GC sensitivity was found in the MLL germline ALL cell line MHH call2 (Figure 3c), as observed in two independent RNA interference experiments.

**Over-expression of BTNL9 increases resistance of *MLL-AF4* rearranged ALL cells to dexamethasone-induced apoptosis**

Next, we cloned the full-length cDNA encoding Myc-tagged *BTNL9* into expression vector pcDNA3.1, and transfected the GC sensitive *MLL-AF4* rearranged ALL cell lines BEL-1 and RSA4;11. In addition BTNL9 was over-expressed in the moderately GC sensitive *MLL* germline ALL cell line NALM-6. As determined by qPCR, the transfection experiments resulted in a significant up-regulation of BTNL9 (Figure 4a).

No difference in the level of apoptosis could be seen in cells transfected with *BTNL9* expressing vector compared to cells transfected with the empty vector in neither of the pre-B ALL cell lines (data not shown). However, after treatment with 1 μM dexamethasone for 6 h and 12 h, the percentage of viable cells was much lower in the GC-sensitive *MLL-AF4* rearranged ALL cells expressing Myc-tagged *BTNL9* compared with cells transfected with empty vector (Figure 3b). However, only a slide but not significant change in apoptosis-level could be seen in the *MLL* germline ALL cell line NALM-6

**Expression of the *BTNL9* in response to other apoptotic inducers**

To gain a better understanding into the regulation of *BTNL9* and to check whether *BTNL9* is generally associated with apoptosis, we screened a panel of cytokines, drugs and stress inducing conditions for effect on *BTNL9* expression level. While none of the stress inducing conditions like hypoxia, serum starvation or heat-shock were able to upregulate *BTNL9*, *BNTL9* expression-level significantly increased after treatment with apoptosis-inducing drugs like etoposide, staurosporine and retinoic acid. In addition, up-regulation of *BTNL9* was triggered by different hormones like progesterone and β-estradiol (Table 1).

The apoptotic stimulators etoposide (a topoisomerase inhibitor) and staurosporine (a protein kinase inhibitor) stimulated *BTNL9* mRNA expression as well in the GC resistant cell line REH (Supplementary Figure 2). REH does not have a functional GR, proving that BTNL9 also is involved in GC-independent apoptosis. In addition, upregulation of *BTNL9* by apoptotic triggers was not limited to pre-B ALL cells, as it was also induced in Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line (Supplementary Figure 2), indicating a general role for BTNL9 in apoptosis.

## Discussion

ALL with rearrangements of the *MLL* gene represents an aggressive, high-risk form of leukemia and is associated with a highly unfavorable clinical outcome. MLL is a transcription factor and functions as a positive regulator of *HOX* gene expression [17]. *MLL* gene translocations involve about 60 partners, the *AF4 (AFF1)* gene on chromosome 4q21 being the most common one. This type of ALL is very common amongst infants under twelve months, with a poor survival rate of less then 50% of cases [18]. Significantly contributing to this poor prognosis is cellular drug resistance, including resistance to L-asparaginase and synthetic GC like dexamethasone and prednisone [19]

GC are key regular components in multi-agent chemotherapy protocols used for the treatment of ALL due to their ability to induce apoptosis in immature pre-B cells and thymocytes. However, resistance to GC is a serious problem in the treatment of all types of ALL, especially in *MLL* rearranged ALL affecting infants. The reason for this resistance is currently unknown but a change in the expression profile is thought to be a main factor in the biologic mechanisms that maintain resistance to these drugs. Recently, high-level of *MCL-1* and *S100A8/S100A9* expression were found to contribute to GC resistance in infant ALL *in vitro* and *in vivo* [20, 21]. However, more genes are thought to be involved in *MLL* rearranged ALL GC resistance.

In this study, we have identified upregulation of *BTNL9* after GC treatment in pre-B ALL and AML cell lines. BTNL9 belongs to the B7 protein super-family and is primarily expressed in primary and secondary lymphoid tissues such as bone marrow, lymph knots, thymus and spleen, as well as in B cells [10]. *BTNL9* expression was rapidly induced after dexamethasone treatment. This rapid induction was blocked when cells were treated with the GR antagonist RU486, pointing the possibility of a direct transactivation through the GR.

Recently, *Kang et al.* have found high expression levels of *BTNL9* associated with high-risk ALL in a panel of 47 cases of infant *MLL-AF4* rearranged ALL [16]. Here we show that ALL cell lines expressing high-levels of *BTNL9* have a poor response to GC-induced apoptosis. Downregulation of BTNL9 protein by

RNA interference, demonstrated a clear increase in dexamethasone sensitivity in *MLL-AF4* rearranged ALL cells and at a lower level in *MLL* germline ALL cells. In line with this findings, overexpression of BTNL9 in GC-sensitive *MLL-AF4* rearranged ALL cells made them more resistant to GC-induced apoptosis.

The fact that low BTNL9 levels not only promote sensitivity to GC treatment to *MLL* rearranged ALL cell lines but also to GC poor responding *MLL* germline ALL cells, might indicate a general role of BTNL9 in GC resistance in pre-B ALL. However, only a slight change could be seen when down-regulating BTNL9 and no significant decrease in GC sensitivity could be observed in GC-sensitive *MLL* germline cells after enforced BTNL9 expression. When genotyping a cohort of 384 pediatric ALL cases [22] for the *BTNL8-BTNL3* CNV, we found a moderate but significant increase *($p = 0.033$* for dexamethasone and *$p = 0.033$* for prednisone) in GC resistant patients homozygous for the *BNTL8_BTNL3-del* wild-type allele (Supplementary Figure 3). Our results show that although *BNTL9* expression levels do not correlate with the *BTNL8-BTNL3* genotype in *MLL* rearranged ALL cells, it seems to correlate in *MLL* germline ALL cells, indicating that BNTL9 may indeed play a role in GC resistant *MLL* germline ALL. However, follow-up experiments and a larger patient cohort will be needed to shed more light on this question.

BTNL9 belongs to the B7 superfamily, a protein family involved in the regulation of T-cell activation and tolerance by providing positive or negative secondary signals [23]. Same as B7 proteins, members of the BTN/BTNL family have been shown to be able to co-stimulate or co-inhibit T-cell activation [24, 25][26-28]. B7 proteins are expressed on a variety of hematopoietic malignancies, solid tumors and tumor-infiltrating immune cells, were they provide the basis for dynamic interactions between tumors and the host immune system [29-31]. Moreover, it has been shown that immature lymphocytes can be ‘rescued’ when these cells express high levels of the co-stimulatory B7-1 or B7-2 molecules [32]. However, so far it is unknown whether BTNL9 has co-stimulatory, co-inhibitory or both properties.

Some co-inhibitory B7 members, like CTLA-4 and B7-H4, mediate tumorigenesis by inhibiting apoptosis through the MAP kinases, ERK, p38 and JNK signaling pathway [33, 34]. GC resistance frequently appears in malignant cells due to aberrant activation of various protein kinases that exert anti-apoptotic effects [35]. One strategy to overcome GC resistance would be to prevent the activities of the PI3K/Akt/mTOR, MEK1/ERK1/2, and other activated protein kinase pathways. In fact, LY294002, a potent PI3K inhibitor recently has been shown to sensitized otherwise resistant MLL rearranged ALL cells [36].

It will be interesting to check whether BTNL9 functions through the ERK/PI3 signaling pathway in a similar way as CTLA-4 and B7-H4. Interestingly, in our study we have shown that BTNL9 induction is not limited to GC. In fact, a wide range of apoptosis stimulating reagents induced BTNL9 expression, suggesting a common role of BTNL9 in apoptosis. Therefore it would be interesting to test an effect of BNTL9 down-regulation in other types of cancer.

Taken together, the present study presents BTNL9 as an attractive target for therapeutic intervention in order to improve the response to GC and with that improve the prognosis for *MLL* rearranged ALL. However, follow-up studies will be needed to examine pathways BTNL9 is involved in.

## Materials and Methods

### Cell culture

Pre-B ALL cell lines and the AML cell line MV4;11, were maintained in RPMI 1640 supplemented with 2 mM glutamine, 10% (v/v) heat-inactivated fetal bovine serum, 100 μg/mL penicillin and 100 μg/mL streptomycin (Invitrogen) and grown as suspension culture at 37ºC in humidified air containing 5% $CO_2$. Dexamethasone was purchased from Sigma and a stock solution was prepared in 100% DMSO. 1 x $10^6$ cells/ml were seeded 24 h before treatment with 1 μM dexamethasone. Control cells were treated with DMSO.

### Real-Time PCR analysis

Total mRNA was extracted from cells using the miRNA easy Kit (Qiagen), samples were treated with DNase I (Qiagen) for 15 min and 1 to 2 μg of RNA was reverse transcribed using the Superscript VILO kit (Invitrogen) according to the manufacturer's protocol. Real time PCR was carried out using the Light cycler 480 from Roche. The PCR reaction contained 100 ng/μl of cDNA, 10 pmol of each of the specific primers and 5 μl SYBR Green master mix in a final reaction volume of 10 μl. All reactions were performed in triplicates. Thermal-cycling conditions for *ACTB and BTNL9* consisted of an initial denaturation of 10 min at 98°C, 40 cycles of 15 s at 95°C denaturation, 15 s annealing at 61°C, and 18 s elongation at 72°C, and a final extension step at 72°C for 10 min. Cumulative fluorescence was measured after each of the 40 cycles. Product specific amplification was confirmed by melting curve analysis. Oligonucleotide sequences used for quantification were as follows: *BTNL9* 5-' AGCAGCCCAAAAATATGCAG-3' as forward primer and 5'-CACGTGCACCTCCCAGTAGT-3' as reverse primer and ACTB 5'-AGAGCTACGAGCCTGCCTGAC-3' as forward and 5'-AAAGCCATGCCAATCTCATC-3' as reverse primer. Relative Quantification of *BTNL9* gene expression was determined by the construction of a relative expression calibration curve using serial dilutions of *ACTB* as a positive control.

### *In vitro* apoptosis assay

*In vitro* dexamethasone cytotoxicity was determined using the Cell Death Detection ELISA$^{PLUS}$ kit (Roche) according to manufacturer's protocol. Briefly, 1 x $10^4$ exponentially growing cells, previously treated with dexamethasone, prednisone or DMSO for the indicated time, were placed into a 96 well plate and incubated for 4 h. Cells were lysed and 20 µl from the supernatant was transferred into streptavidin coated MP for analysis. Next, Immunoreagent was added, MP was covered and incubated for 2 h at RT. Solution was removed and wells were rinsed with ABTS solution. 100 µl Stop solution was added and samples were measured at 405 nm.

### RNA interference

BTNL9 knock-down were performed in the dexamethasone-resistant ALL cell lines MHH call2 and SEM and the dexamethasone-resistant AML cell line MV4;11. Cells were transfected using the Amaxa® Nucleofector® Transfection System (Lonza) and the Amaxa® Cell Line Nucleofector® Kit R (Lonza) according to manufacturer's protocol. Briefly, one day prior transfection, cells were washed and medium was changed to keep cells in log-growth phase. The next day, first, Nucleofector solution was prepared, contained 300 nM *BTNL9* or a validated nonsilencing control siRNA (specify!!) and 2 µg GPF vector. ON-TARGETplus technology from Thermo scientific for used for this experiment. Target sequence used for *siBTNL9* was: GCUCAAAACGUGACGGCAA. Next, 1 x $10^7$ cells/ml were counted, centrifuged and resuspended in 100 µl Nucleofector solution. Program T-016 was selected on Nucleofector device, samples were pulsed and placed in 2 ml RPM1 medium as described previously and grown for 24 h. To obtain a pure population of transfected cells, cells were sorted by flow-cytometry for GFP in a live cell-sorting device after 24 h and placed again in 2ml RPM1 medium and let grow for 12 h. After that cells were treated with 1µl dexamethasone for another 12 h. Transfected cells were then analyzed for BTNL9 expression by western-blot.

**Over-expression**

Full-length cDNA of human *BTNL9* was cloned into a mammalian expression vector pcDNA$^{TM}$ 3.1(+)/myc-HisC (life technologies) using the restriction enzymes *HindIII* and *EcoRV*. Next, the GC-sensitive cell lines BEL-1, NALM-6 and RSA4;11 were transfected with the Amaxa® Nucleofector® Transfection System (Lonza) as descriped above using 1 μg expression vector, after 24h cells were treated with Neomycin to obtain a 100% transfected population.

**Western blot**

Cell lysates were prepared as previously described [37]. Briefly, equal amounts of proteins (300–350 μg) were resolved by NuPAGE (4-12%; Invitrogen) and transferred to nitrocellulose membranes. Proteins were then blocked by incubation in 10% dry milk in TBST (0.1% Tween-20 in TBS) and probed with the indicated Antibody. Rabbit anti-BTNL9 antibody (ab87049; Abcam) was used in a 1:1000 dilution and incubated for 24 h at 4ºC. Mouse anti-Tubulin antibody was purchased from Santa Cruz (sc-5286). Blots were then developed by enhanced chemiluminescence (ECL; Amersham).

**Statistical analysis**

Differences in *BTNL9* gene expression between cell lines were statistically evaluated using the student *t*-test or the Mann-Whitney *U*-test. Differences were considered statistically significant at *p-values <0.05*.

## References


1. Schultz KR, Pullen DJ, Sather HN, Shuster JJ, Devidas M, Borowitz MJ, Carroll AJ, Heerema NA, Rubnitz JE, Loh ML, et al: **Risk- and response-based classification of childhood B-precursor acute lymphoblastic leukemia: a combined analysis of prognostic markers from the Pediatric Oncology Group (POG) and Children's Cancer Group (CCG).** *Blood* 2007, **109:**926-935.
2. Pui CH: **Toward a total cure for acute lymphoblastic leukemia.** *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2009, **27:**5121-5123.
3. Felix CA, Lange BJ: **Leukemia in infants.** *The oncologist* 1999, **4:**225-240.
4. Sam TN, Kersey JH, Linabery AM, Johnson KJ, Heerema NA, Hilden JM, Davies SM, Reaman GH, Ross JA: **MLL gene rearrangements in infant leukemia vary with age at diagnosis and selected demographic factors: a Children's Oncology Group (COG) study.** *Pediatric blood & cancer* 2012, **58:**836-839.
5. Esposito G, Cevenini A, Cuomo A, de Falco F, Sabbatino D, Pane F, Ruoppolo M, Salvatore F: **Protein network study of human AF4 reveals its central role in RNA Pol II-mediated transcription and in phosphorylation-dependent regulatory mechanisms.** *The Biochemical journal* 2011, **438:**121-131.
6. Huret JL, Dessen P, Bernheim A: **An atlas of chromosomes in hematological malignancies. Example: 11q23 and MLL partners.** *Leukemia* 2001, **15:**987-989.
7. Daser A, Rabbitts TH: **The versatile mixed lineage leukaemia gene MLL and its many associations in leukaemogenesis.** *Seminars in cancer biology* 2005, **15:**175-188.
8. Dordelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, Gadner H, Riehm H, Schrappe M: **Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia.** *Blood* 1999, **94:**1209-1217.
9. Spokoini R, Kfir-Erenfeld S, Yefenof E, Sionov RV: **Glycogen synthase kinase-3 plays a central role in mediating glucocorticoid-induced apoptosis.** *Mol Endocrinol* 2010, **24:**1136-1150.
10. Arnett HA, Escobar SS, Viney JL: **Regulation of costimulation in the era of butyrophilins.** *Cytokine* 2009, **46:**370-375.
11. Aigner J, Villatoro S, Rabionet R, Roquer J, Jimenez-Conde J, Marti E, Estivill X: **A common 56-kilobase deletion in a primate-specific segmental duplication creates a novel butyrophilin-like protein.** *BMC genetics* 2013, **14:**61.
12. Blenk S, Engelmann J, Weniger M, Schultz J, Dittrich M, Rosenwald A, Muller-Hermelink HK, Muller T, Dandekar T: **Germinal center B cell-like (GCB) and activated B cell-like (ABC) type of diffuse large B cell lymphoma (DLBCL): analysis of molecular predictors, signatures, cell cycle state and patient survival.** *Cancer informatics* 2007, **3:**399-420.
13. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, et al: **Distinct types of diffuse large B-cell**

**lymphoma identified by gene expression profiling.** *Nature* 2000, **403:**503-511.

14. Schmidt S, Rainer J, Riml S, Ploner C, Jesacher S, Achmuller C, Presul E, Skvortsov S, Crazzolara R, Fiegl M, et al: **Identification of glucocorticoid-response genes in children with acute lymphoblastic leukemia.** *Blood* 2006, **107:**2061-2069.
15. Bhadri VA, Cowley MJ, Kaplan W, Trahair TN, Lock RB: **Evaluation of the NOD/SCID xenograft model for glucocorticoid-regulated gene expression in childhood B-cell precursor acute lymphoblastic leukemia.** *BMC Genomics* 2011, **12:**565.
16. Kang H, Wilson CS, Harvey RC, Chen IM, Murphy MH, Atlas SR, Bedrick EJ, Devidas M, Carroll AJ, Robinson BW, et al: **Gene expression profiles predictive of outcome and age in infant acute lymphoblastic leukemia: a Children's Oncology Group study.** *Blood* 2012, **119:**1872-1881.
17. de Boer J, Walf-Vorderwulbecke V, Williams O: **In focus: MLL-rearranged leukemia.** *Leukemia* 2013, **27:**1224-1228.
18. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, Hovi L, LeBlanc T, Szczepanski T, Ferster A, et al: **A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial.** *Lancet* 2007, **370:**240-250.
19. Muntean AG, Hess JL: **The pathogenesis of mixed-lineage leukemia.** *Annual review of pathology* 2012, **7:**283-301.
20. Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, de Lorenzo P, Sallan SE, Brady HJ, Armstrong SA, Pieters R: **Association of high-level MCL-1 expression with in vitro and in vivo prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia.** *Blood* 2010, **115:**1018-1025.
21. Spijkers-Hagelstein JA, Schneider P, Hulleman E, de Boer J, Williams O, Pieters R, Stam RW: **Elevated S100A8/S100A9 expression causes glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia.** *Leukemia* 2012, **26:**1255-1265.
22. Nordlund J, Backlin CL, Wahlberg P, Busche S, Berglund EC, Eloranta ML, Flaegstad T, Forestier E, Frost BM, Harila-Saari A, et al: **Genome-wide signatures of differential DNA methylation in pediatric acute lymphoblastic leukemia.** *Genome Biol* 2013, **14:**r105.
23. Seliger B, Marincola FM, Ferrone S, Abken H: **The complex role of B7 molecules in tumor immunology.** *Trends in molecular medicine* 2008, **14:**550-559.
24. Smith IA, Knezevic BR, Ammann JU, Rhodes DA, Aw D, Palmer DB, Mather IH, Trowsdale J: **BTN1A1, the mammary gland butyrophilin, and BTN2A2 are both inhibitors of T cell activation.** *J Immunol* 2010, **184:**3514-3525.
25. Kanneganti V, Kama R, Gerst JE: **Btn3 is a negative regulator of Btn2-mediated endosomal protein trafficking and prion curing in yeast.** *Molecular biology of the cell* 2011, **22:**1648-1663.
26. Arnett HA, Escobar SS, Gonzalez-Suarez E, Budelsky AL, Steffen LA, Boiani N, Zhang M, Siu G, Brewer AW, Viney JL: **BTNL2, a butyrophilin/B7-like**

**molecule, is a negative costimulatory molecule modulated in intestinal inflammation.** *J Immunol* 2007, **178:**1523-1533.

27. Nguyen T, Liu XK, Zhang Y, Dong C: **BTNL2, a butyrophilin-like molecule that functions to inhibit T cell activation.** *J Immunol* 2006, **176:**7354-7360.
28. Chapoval AI, Smithson G, Brunick L, Mesri M, Boldog FL, Andrew D, Khramtsov NV, Feshchenko EA, Starling GC, Mezes PS: **BTNL8, a butyrophilin-like molecule that costimulates the primary immune response.** *Molecular immunology* 2013, **56:**819-828.
29. Thompson RH, Dong H, Kwon ED: **Implications of B7-H1 expression in clear cell carcinoma of the kidney for prognostication and therapy.** *Clinical cancer research : an official journal of the American Association for Cancer Research* 2007, **13:**709s-715s.
30. Krambeck AE, Thompson RH, Dong H, Lohse CM, Park ES, Kuntz SM, Leibovich BC, Blute ML, Cheville JC, Kwon ED: **B7-H4 expression in renal cell carcinoma and tumor vasculature: associations with cancer progression and survival.** *Proc Natl Acad Sci U S A* 2006, **103:**10391-10396.
31. Zang X, Thompson RH, Al-Ahmadie HA, Serio AM, Reuter VE, Eastham JA, Scardino PT, Sharma P, Allison JP: **B7-H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome.** *Proc Natl Acad Sci U S A* 2007, **104:**19458-19463.
32. Wagner DH, Jr., Hagman J, Linsley PS, Hodsdon W, Freed JH, Newell MK: **Rescue of thymocytes from glucocorticoid-induced cell death mediated by CD28/CTLA-4 costimulatory interactions with B7-1/B7-2.** *The Journal of experimental medicine* 1996, **184:**1631-1638.
33. Qian Y, Hong B, Shen L, Wu Z, Yao H, Zhang L: **B7-H4 enhances oncogenicity and inhibits apoptosis in pancreatic cancer cells.** *Cell and tissue research* 2013, **353:**139-151.
34. Zhao X, Zhang GB, Gan WJ, Xiong F, Li Z, Zhao H, Zhu DM, Zhang B, Zhang XG, Li DC: **Silencing of B7-H3 increases gemcitabine sensitivity by promoting apoptosis in pancreatic carcinoma.** *Oncology letters* 2013, **5:**805-812.
35. Thompson EB: **Stepping stones in the path of glucocorticoid-driven apoptosis of lymphoid cells.** *Acta biochimica et biophysica Sinica* 2008, **40:**595-600.
36. Spijkers-Hagelstein JA, Pinhancos SS, Schneider P, Pieters R, Stam RW: **Chemical genomic screening identifies LY294002 as a modulator of glucocorticoid resistance in MLL-rearranged infant ALL.** *Leukemia* 2013.
37. Minones-Moyano E, Porta S, Escaramis G, Rabionet R, Iraola S, Kagerbauer B, Espinosa-Parrilla Y, Ferrer I, Estivill X, Marti E: **MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function.** *Hum Mol Genet* 2011, **20:**3067-3078.

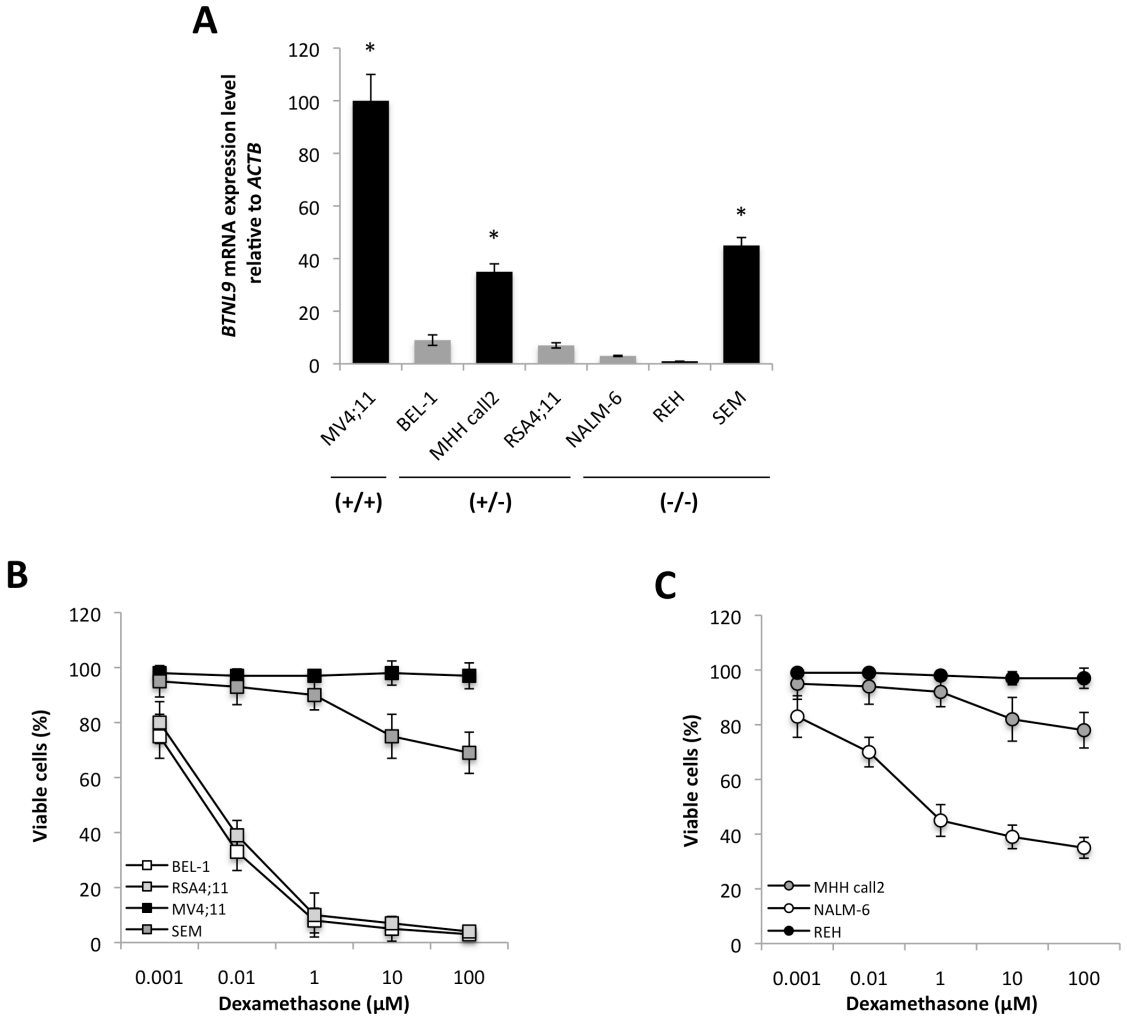



**Figure 1. *BTNL9* expression in pre-B ALL cell lines.** (A) Cell lines were genotyped for the *BTNL8_BTNL3-del* allele and *BTNL9* mRNA expression was measured by qPCR. MV4;11 is homozygous for the wild-type allele, BEL-1, MHH call 2 and RSA4;11 are heterozygous, and NALM-6, REH and SEM are homozygous for the *BTNL8-BTNL3* deletion CNV. *In vitro* dexamethasone response in (B) *MLL-AF4* rearranged ALL and (C) *MLL* germline ALL cell lines. (A) MHH call2, MV4;11 and SEM are resistant to dexamethasone (black bars), and BEL-1, RSA4;11 and NALM-6 are dexamethasone sensitive (grey bars).

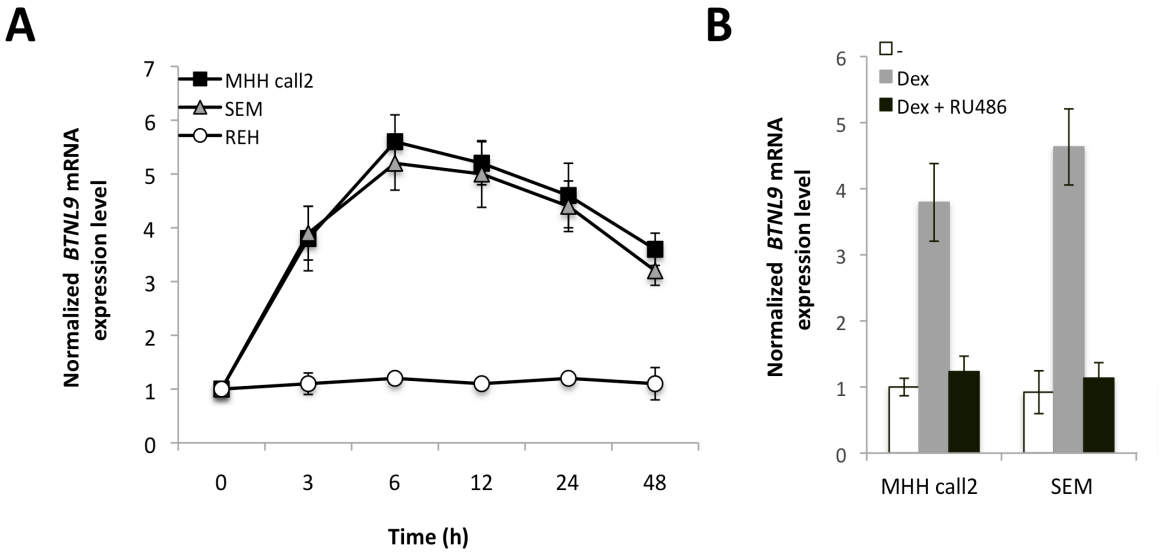



**Figure 2. Dexamethasone-induced upregulation of *BTNL9*.** (A) The expression of *BTNL9* was immediately induced after treatment with 1 μM dexamethasone in the pre-B ALL cell lines MHH call2 and SEM. In contrary, no upregulation of *BTNL9* could be observed in the GC resistant cell line REH, indicating that BTNL9 induction depends on a functional GR. (B) RU486, a GR antagonist, prevented the induction of *BTNL9* by dexamethasone. Pre-B ALL cells were treated with DMSO (control), 1 μM dexamethasone or 1 μM dexamethasone plus 10 μM RU486.

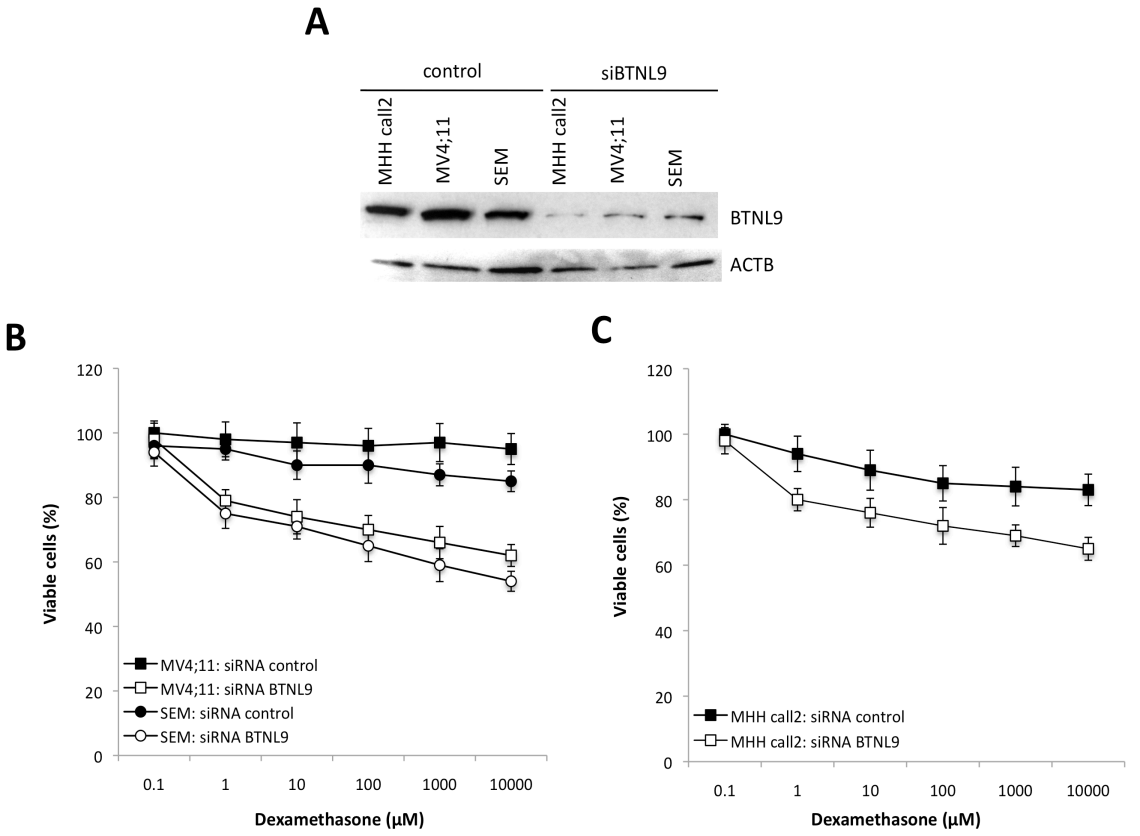



**Figure 3. Downregulation of BTNL9 sensitizes both, dexamethasone-resistant *MLL* rearranged ALL and *MLL* germline ALL cell lines.** (A) siRNA experiments directed against human BTNL9 into dexamethasone-resistant SEM or MV4;11 cells showed suppression in BTNL9 protein expression compared with control cells (transfected with scrambled siRNA). The effects of BTNL9 downregulation on the *in vitro* dexamethasone response on (B) SEM and MV4;11 or (C) MHH call2 cells were assessed by a photometric enzyme-immunoassay for determination of cytoplasmic histone-associated DNA-fragments after induced cell death, performed in triplicate. The graph shows the mean dexamethasone response curves in cells transfected with either control or a siRNA against human BTNL9, derived from two independent RNA interference experiments.

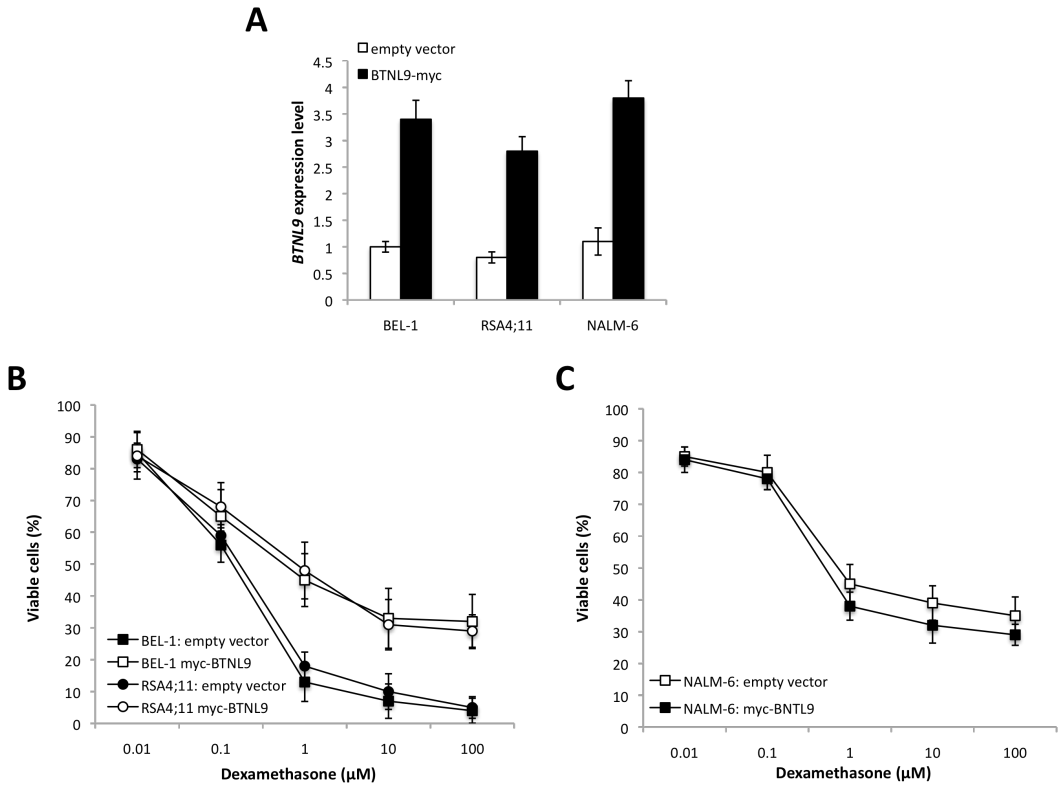
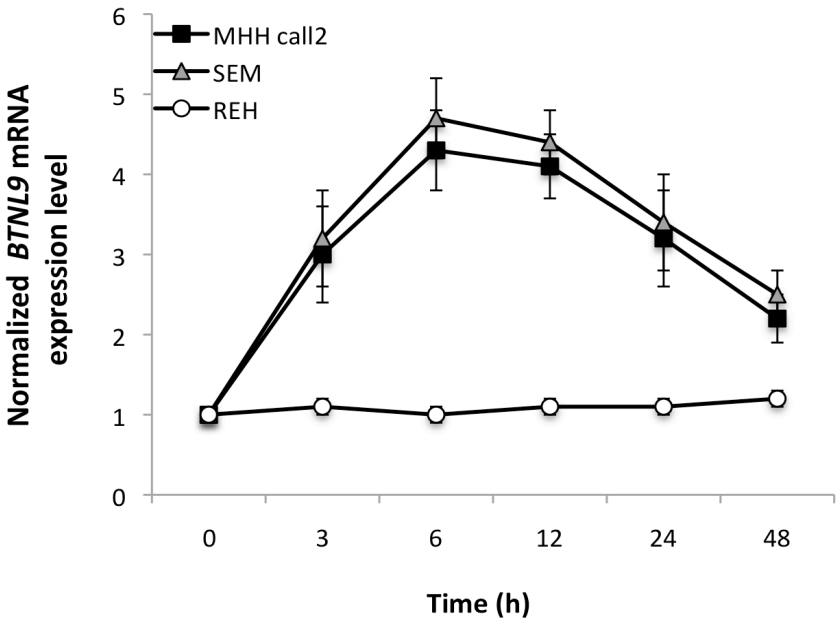


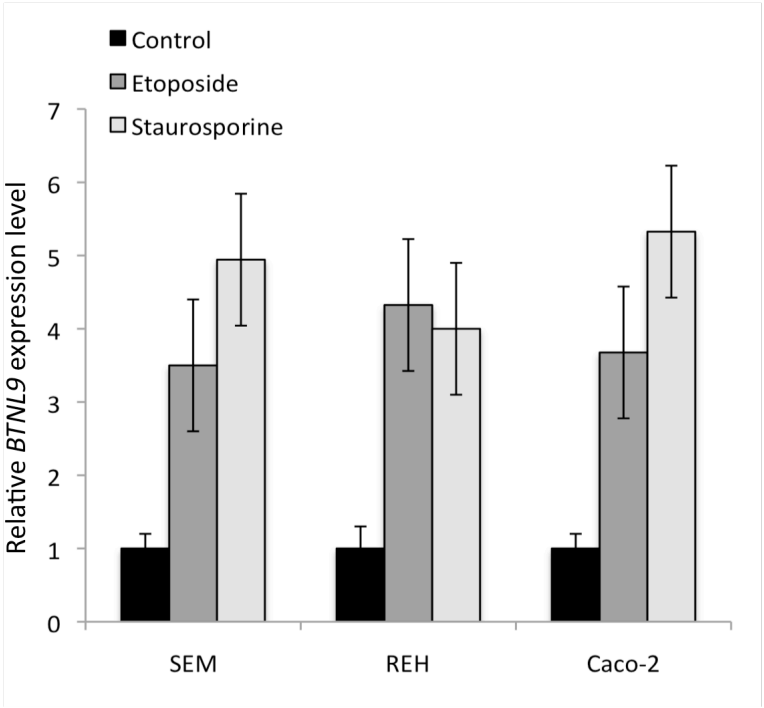

**Figure 4. BTNL9 over-expression induces dexamethasone resistance in *MLL-AF4* rearranged ALL cells.** (A) qPCR analysis showing mRNA expression of BTNL9 in the dexamethasone-sensitive cell lines BEL-1, RSA4;11 and NALM-6 transduced with a mammalian expression vector encoding human BTNL9. (B) In vitro dexamethasone response of BEL-1, RSA4;11 and (C) NALM-6 in the absence and presence of enforced BTNL9 expression.

**Table 1.** Panel of various pharmacological agents were screened in pre-B ALL cells for their effects on *BTNL9* gene expression

| Treatment | up-regulate *BTNL9*? | Treatment | up-regulate *BTNL9*? |
|---|---|---|---|
| IL1-β | No | Hypoxia | No |
| TNFα | No | Prednisone | Yes |
| LPS | No | Dexamethasone | Yes |
| β-Estradiol | No | Etoposide | Yes |
| Progesterone | Yes | Staurosporine | Yes |
| Retinoic acid | Yes | NaCl | No |
| DMSO | No | Heatshock | No |
| Serum starvation | No | beta-Mercaptoethanol | No |
| UV | Yes | | |




**Supplementary figure 1. Prednisone-induced upregulation of *BTNL9*.** Same as with the glucocortidoid dexamethasone, treatment with 1 µl prednisone immediately induced expression of *BTNL9* in the pre-B ALL cell lines MHH call2 and SEM. No upregulation of *BTNL9* could be observed in the GC resistant cell line REH, indicating that *BTNL9* induction depends on a functional GR.




**Supplementary figure 2:** After treatment with the apoptotic inducers etoposide and staurosporine for 6 h, *BTNL9* expression level was induced in REH as well as in Caco-2, suggesting a GR-independent role of BTNL9 in apoptosis.

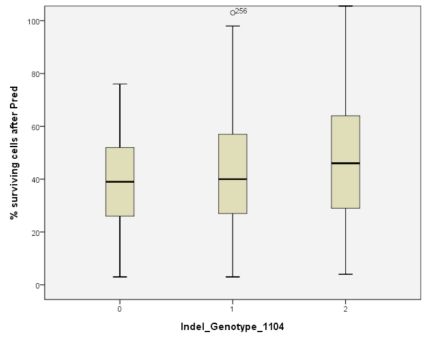
**Prednisolone *P* = 0.033**

**Case Processing Summary**

| Indel_Genotype_1104 | | Cases | | | |
|---|---|---|---|---|---|
| | | Valid | | Missing | |
| | | N | Percent | N | Percent |
| deb::Pred_50 | 0 | 53 | 98,1% | 1 | 1,9% |
| | 1 | 157 | 98,7% | 2 | 1,3% |
| | 2 | 161 | 94,2% | 10 | 5,8% |

**Case Processing Summary**

| Indel_Genotype_1104 | | Cases | |
|---|---|---|---|
| | | Total | |
| | | N | Percent |
| deb::Pred_50 | 0 | 54 | 100,0% |
| | 1 | 159 | 100,0% |
| | 2 | 171 | 100,0% |



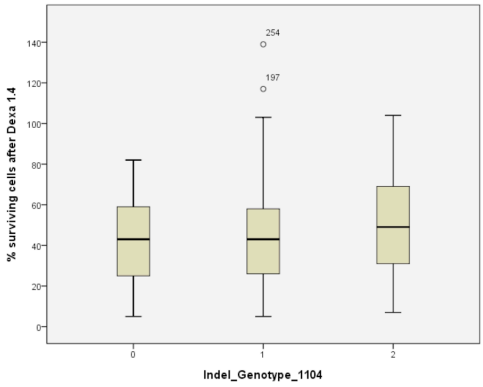

**Dexametasone *P* = 0.033**

**Case Processing Summary**

| Indel_Genotype_1104 | | Cases | | | |
|---|---|---|---|---|---|
| | | Valid | | Missing | |
| | | N | Percent | N | Percent |
| deb::Dexa_1_4 | 0 | 54 | 100,0% | 0 | ,0% |
| | 1 | 158 | 99,4% | 1 | ,6% |
| | 2 | 161 | 94,2% | 10 | 5,8% |

**Case Processing Summary**

| Indel_Genotype_1104 | | Cases | |
|---|---|---|---|
| | | Total | |
| | | N | Percent |
| deb::Dexa_1_4 | 0 | 54 | 100,0% |
| | 1 | 159 | 100,0% |
| | 2 | 171 | 100,0% |




**Supplementary figure 3:** Association of the *BTNL8_BTNL3-del* allele with glucocorticoid-resistance in a cohort of 384 pediatric pre-B ALL samples *in vivo.*

# DISCUSSION

The *BTNL* family consists of four genes. However, up to this date only one of them, *BTNL2*, has been characterized [46]. Like other genes of the closely related B7 family, *BTNL2* has been shown to be involved in immunoregulatory control of immune-related disorders [59, 63-65]. In addition, polymorphism in the human *BTNL2* gene have been linked to inflammatory diseases and *BTNL2* has been shown to act as a coinhibitory molecule for T-cell activation [49, 50].

In order to gain a better understanding about this gene family, in this thesis, we undertook a comprehensive analysis of the remaining three *BTNL* genes, *BTNL3*, *BTNL8* and *BTNL9*. All three genes are located in a cluster at the subtelomeric region of human chromosome 5q35.3, together with several genes encoding tripartite motif-containing (TRIM) proteins, and genes involved in the olfactory system.

First, we looked at the genomic level and structurally described a previously uncharacterized 56 kb deletion polymorphism, located between two 1.6 kb long low copy repeats (LCR) with 98% sequence identity of two primate-specific genes, namely *BTNL8* and *BTNL3* [122]. LCRs are frequently associated with genomic rearrangements, usually resulting from non-allelic homologous recombination (NAHR) events [123, 124]. In the human genome, CNVs are a major source of genetic variation and have been increasingly studied for disease association [125]. Particularly in genes playing a role in defense and immune response CNV regions are highly enriched, indicating a link between CNVs and human health [126]. So have many CNVs affecting genes or other functional elements, such as promoters or enhancers, have been found to play important roles in several disorders, including autoimmune, neuropsychiatric and infectious diseases and cancer [127].

The *BTNL8-BTNL3* deletion CNV affects two primate-specific genes, *BTNL8* and *BTNL3*, who share 80% homology in their coding sequence, and leads to the formation of a novel *BTNL8*3* hybrid gene consisting of the first five exons of *BTNL8* and the last three exons of *BTNL3*. Next, an antibody against the C-terminal end of BTNL3 was developed and the existence of a fusion BTNL8*3 protein was confirmed in lymphoblastoid cell lines (LCLs), heterozygous or

homozygous for the *BTNL8_BTNL3-del* allele without any alterations in the reading frame. The encoded BTNL8*3 fusion protein contains the N-terminal portion of BTNL8 encoding a IgV, IgC and a transmembrane domain and a B30.2 domain of BTNL3 at its C-terminal end. However, it remains to be assessed if and to with extend this new chimeric BTNL8*3 protein can compensate for the function of the BTNL8 and BTNL3 wild-type molecules.

The novel *BTNL8*3* hybrid gene is under the influence of the *BTNL8* promoter and its functional elements. However, as we could show by allele-specific PCR, the *BTNL8*3* gene was expressed at a significantly lower level, compared to wild-type *BTNL8,* in several tissues heterozygous for *BTNL8_BTNL3-del*, which could be due to a less stable mRNA product.

As mentioned above, up to this date little is known about the function of *BTNL3* and *BTNL8*. However, it has been shown that *BTNL3* and *BTNL8* are primarily expressed by tissues of the digestive tract. Recently, murine *Btnl1* has been identified to regulate interactions with intraepithelial γδ T-lymphocytes in the murine small intestine [52]. In line with these findings, one could speculate about a function for *BTNL3* and *BTNL8* in the immune response of human mucosal epithelia. Epithelia are primary targets of bacterial and viral infection and act as one of the key barriers in our immune system [128]. A diversity of innate and adaptive immune cells play a role in the detection and elimination of invading pathogens. However, in the healthy intestine, mucosal immune cells have to discriminate between potentially harmful and beneficial antigens. Dysregulation of this balance can result in inflammatory disease and associated carcinoma [129, 130]. Similar to *Btnl1* in mice, *BTNL3* and *BTNL8* could play a role in the suppression of the activation of intraepithelial γδ T-lymphocytes. A suppression of their activation by *BTNL8* or *BTNL3* could result in a control of cancer progression by T-lymphocytes and a prevention of an excessive immune response. However, this hypothesis will have to be investigated.

Next, we developed a PCR-based genotyping assay and genotyped 1,103 samples from 11 HapMap populations, 1,007 samples derived from 39 ethnical groups of the Centre d'Etude du Polymorphisme Humain (CEPH) Human

Genome Diversity Panel (HGDP) and 477 Spanish samples for the *BTNL8_BTNL3-del allele*. We found significant differences in the stratification of the deletion variant. The deletion is very rare in African and Oceanic and Middle Eastern population and common in European, American and East Asian population. Ethnicity plays an important role in inter-individual variability of the immune system. It has been shown that certain ethnic groups which are under constant exposure to different pathogens, have selected genetic adaptations that provide resistance or reduced susceptibility to infection, meaning that for some populations CNVs can result in an advantageous phenotype [131]. The first gene identified, where a reduction in copy number was shown beneficial was the α-globin locus, where it increases resistance to malaria infection and susceptibility to mild α-thalassemia [132]. Other examples where the number of gene copies positively correlates with infection are *FCGR3B* and *DEFB4* genes, which are associated with glomerulonephritis, and Crohn's disease [133, 134], respectively. The marked population differences found of *BTNL8_BTNL3-del* frequencies suggest that this deletion CNV might have evolved under positive selection due to environmental conditions in some populations, with potential phenotypic consequences. In addition, ethnical differences result in variability to treatment outcome for many drugs [131]. Given the big impact of B7 proteins in the immune system and immune-related diseases, the *BTNL8-BTNL3* deletion variant could be interesting for both, pharmacogenomics and individualized drug therapy in the future [96].

After a detailed linkage disequilibrium analysis for single nucleotide polymorphism (SNPs) in the genomic region surrounding the *BTNL8-BTNL3* CNV, we only were able to identify a suitable tag SNP (LD $r^2$>0.8) only in northern European, American and Asian populations. However, no SNP could be identified in southern European and African population that could serve as a surrogate for the deletion variant. African population have been shown to differ significantly in their haplotype and LD structure from other ethnical groups. Moreover, southern European groups such as Spanish, Greek and Italians constantly are under the influence of migratory influences and admixture with

other ethnical group from Africa and northern Europe, what could result in a LD structure different from other European populations [135, 136].

In a next step, we undertook an expression analysis and looked at genome-wide expression data produced by *Stranger et al., 2005* [137]. With this strategy, we were able to identify several genes whose expression-level was affected by the *BTNL8-BTNL3* deletion CNV. The 20 genes validated by qPCR in LCLs homozygous for *BTNL8_BTNL3-del*, were submitted to Ingenuity Pathway Analysis (IPA) program for functional classification and to check whether these genes interact with each other in regulatory networks and biological pathways. With this strategy, one well-defined network with TNF and the ERK1/AKT pathway as central hubs could be identified. TNF, ERK1 and AKT are important players in signal transduction pathways and key components of the immune response in humans, therefore even a slight deregulation of those proteins might have an important impact in the response to pathogens [138]. However, LCLs might not be the main cell type where the *BTNL8*3* CNV affects expression levels, since *BTNL3* and *BTNL8* are predominantly expressed in the digestive tract. Follow-up studies using other cell types will be needed.

In addition, we found major differences in the expression-level of *BTNL9*, another gene of the same family, located ~100 kb from the deletion. Even though none of the regulatory elements of its promoter was affected by the CNV, *BTNL9* mRNA and protein level was significantly lower in LCLs containing one or two copies of *BTNL8_BTNL3-del*. However, it is a well known fact that genomic neighborhoods can influence the expression level of genes by a so called 'positional effect'. This can be achieved by affecting *cis*-regulatory elements, such as transcription factor binding sites, or by re-organization of chromosomes into territories within the nucleus [136].

In the second part of this thesis, we screened existing literature for possible biological functions of *BTNL3*, *BTNL8* and *BTNL9*. Although not much literature was available for neither of the genes, we found several articles showing a de-regulation of the *BTNL9* gene in different hematopoietic malignancies, including

germinal center B-cell like (GBC) type of diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) [139, 140]. In addition, several publications found *BTNL9* to be differentially expressed in pre B-ALL after glucocorticoid (GC) treatment [141, 142] .

To confirm *BTNL9* as a GC-sensitive gene, we treated several pre-B ALL cell lines with the synthetic GC dexamethasone. In all cell lines, with the exception of REH, we found *BTNL9* elevated immediately within 3 hours. This rapid enhancement of expression and the fact that *BTNL9* expression was not induced in REH, a well-characterized cell line with a defective glucocorticoid receptor (GR) [143], suggested that *BTNL9* expression is a primary target of the GR. This hypothesis could be confirmed by the use of the GR antagonist RU486. *BTNL9* expression was completely abolished when cells were co-treated with dexamethasone and RU846. GCs are essential components in the treatment of ALL due to their ability to induce apoptosis in immature lymphoblasts [144]. A good response to introductory GC treatment predicts favorable outcome [120]. However, several types of ALL show a high resistance against GC treatment [145] therefore identifying genes involved in GC-dependent apoptosis is essential to improve outcome in ALL therapy.

In a next step we measured the expression-level of endogenous *BTNL9* in pre-B ALL cell lines and found that *BTNL9* expression significantly varies between cell lines. To check whether in line with previous findings on LCLs, *BTNL9* expression-level is affected by the *BTNL8-BTNL3* deletion CNV, we genotyped all pre-B ALL cell lines for the *BTNL8_BTNL3-del* allele. However, correlation was only found for cell lines containing the germline *MLL* gene. In contrast *MLL* rearranged cell lines showed a significantly reduced (RSA4;11, BEL-1) or significantly elevated (SEM, MV4;11) *BTNL9* expression. *MLL* rearranged ALL is a very aggressive form of ALL with a very bad overall survival rate [146]. This form of leukemia is very common in infants less than one year of age, and it is thought to develop *in utero* [147]. The poor outcome is mainly due to drug resistance, in particular to synthetic GCs [148]. Therefore, in order to improve prognosis it is important to find genes that contribute to GC resistance.

Interestingly, recently high-level *BTNL9* was found to correlate with high-risk in a cohort of *MLL-AF4* rearranged infant ALL cases *in vivo* [145]. In line, all pre-B ALL cell lines used in this thesis expressing high-level *BTNL9* were GC-resistant, while all cell lines expressing low-level *BTNL9* were GC-sensitive. Moreover, downregulation of the BTNL9 protein by RNAi, led to a clear increase in GC sensitivity in *MLL-AF4* rearranged ALL cells and at a reduced level in *MLL* germline ALL cells, and overexpression of BTNL9 in GC-sensitive *MLL-AF4* rearranged ALL cells made them more resistant to GC-induced apoptosis.

To our knowledge, this is the first time that a BTN molecule is associated with apoptosis. However, several B7 homologous have been reported to mediate tumorigenesis by inhibiting apoptosis through ERK1/2 signaling [149]. GC therapy affects the activity of several protein kinases and, vice versa many protein kinases, e.g. PI3K, Akt, mTOR, ERK1/2 and other activated protein kinase pathways, can affect GC-induced apoptosis. One hypothesis is that BTNL9 acts in the same way as PD-1, BH-3 or B7x by inhibiting ERK1/2, what could be in a GR dependent as well as GR independent manner. In fact, when screening a panel of apoptotic stimuli, we found *BTNL9* expression to be increased by a big variety of drugs, indicating a general role of BTNL9 in apoptosis. Moreover, as discussed above expression of the ERK1/AKT pathway was shown to be affected the *BTNL8-BTNL3* deletion CNV, supporting the hypothesis that BTNL9 plays a role in this signal transduction pathway. However, this remains to be elucidated.

Another big question to answer is whether BTNL9 also is involved in GC-resistance of *MLL* germline ALL or in other forms of ALL. *MLL* rearranged ALL and *MLL* germline ALL are thought to have distinct biological entities [150]. However, when genotyping a panel of childhood pre-B ALL patients for the *BTNL8_BTNL3-del* allele we found a slight but significant association of the CNV with GC-resistance. However, this result will have to be validated in another ALL patient cohort. In addition, it would be interesting to check whether *BTNL9* expression-level independent of the *BTNL8-BTNL3* CNV associated with GC-resistance in other forms of ALL.

Taken together, in this thesis we provide a unique, broad, functional analysis of several, up to this date uncharacterized, BTNL family members. We characterize a common CNV with clear functional consequences. Moreover, we identify a completely unexpected role for BTNL9 in *MLL-AF4* rearranged ALL. This finding could be of importance for the development of new therapeutic intervention in *MLL-AF4* rearranged ALL in the future. However, many questions remain unanswered but certainly the BTNL family will acquire much attention during the next years in the field of anti-tumor immunity.

# CONCLUSIONS

The following conclusions can be drawn from the results presented in this thesis:

1. We identified a 56-kb deletion copy number variant (CNV) on human chromosome 5q35.3 that affects two genes *BTNL8* and *BTNL3*.
2. The deletion CNV results in the formation of a novel *BTNL8*3* chimeric gene and mRNA, that translates into a new BTNL8*3 protein.
3. The deletion is covered by several tagging SNPs in northern European, Asian and American population, but tagSNPs are missing in African and southern European populations.
4. Significant population-based differences exist for the *BTNL8_BTNL3-del* allele between major continental groups. The CNV is very common in European, Asian and American population but rare in African and Oceanic population.
5. Lymphoblastoid cell lines containing the *BTNL8_BTNL3-del* allele show a significant reduced expression of *BTNL9*, what might be the result of a "positional effect".
6. The *BTNL8*3* CNV interferes with the expression level of several genes involved in immune response and cancer.
7. *BTNL9* is upregulated in acute lymphocytic leukemia (ALL) cell lines after glucocorticoid treatment, and in different cell systems after treatment with various apoptotic stimuli.
8. Upregulation of *BTNL9* depends on a functional glucocorticoid receptor, and repression of endogenous *BTNL9* led to a higher sensitivity to glucocorticoid treatment.
9. High-level of BTNL9 is associated with a poor glucocorticoid receptor response in *MLL-AF4* rearranged ALL cell lines.
10. Over-expression of *BTNL9* induced glucocorticoid response in glucocorticoid-sensitive *MLL-AF4* rearranged ALL cell lines.

# BIBLIOGRAPHY

1. Ceeraz S, Nowak EC, Noelle RJ: **B7 family checkpoint regulators in immune regulation and disease.** *Trends in immunology* 2013.

2. Greenfield EA, Nguyen KA, Kuchroo VK: **CD28/B7 costimulation: a review.** *Critical reviews in immunology* 1998, **18:**389-418.

3. Rulifson IC, Sperling AI, Fields PE, Fitch FW, Bluestone JA: **CD28 costimulation promotes the production of Th2 cytokines.** *J Immunol* 1997, **158:**658-665.

4. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, Kawai K, Ohashi PS, Thompson CB, Mak TW: **Differential T cell costimulatory requirements in CD28-deficient mice.** *Science* 1993, **261:**609-612.

5. Seliger B, Quandt D: **The expression, function, and clinical relevance of B7 family members in cancer.** *Cancer immunology, immunotherapy : CII* 2012, **61:**1327-1341.

6. Flies DB, Chen L: **The new B7s: playing a pivotal role in tumor immunity.** *J Immunother* 2007, **30:**251-260.

7. Collins M, Ling V, Carreno BM: **The B7 family of immune-regulatory ligands.** *Genome Biol* 2005, **6:**223.

8. Greaves P, Gribben JG: **The role of B7 family molecules in hematologic malignancy.** *Blood* 2013, **121:**734-744.

9. Acuto O, Mise-Omata S, Mangino G, Michel F: **Molecular modifiers of T cell antigen receptor triggering threshold: the mechanism of CD28 costimulatory receptor.** *Immunological reviews* 2003, **192:**21-31.

10. Chambers CA, Kuhns MS, Egen JG, Allison JP: **CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy.** *Annual review of immunology* 2001, **19:**565-594.

11. Seliger B, Marincola FM, Ferrone S, Abken H: **The complex role of B7 molecules in tumor immunology.** *Trends in molecular medicine* 2008, **14:**550-559.

12. Chambers CA, Allison JP: **Costimulatory regulation of T cell function.** *Current opinion in cell biology* 1999, **11:**203-210.

13. Greenwald RJ, Freeman GJ, Sharpe AH: **The B7 family revisited.** *Annual review of immunology* 2005, **23:**515-548.

14. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S: **CTLA-4 control over Foxp3+ regulatory T cell function.** *Science* 2008, **322:**271-275.

15. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP: **Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies.** *The Journal of experimental medicine* 2009, **206:**1717-1725.

16. Sun L, Wu J, Yi S: **Foxp3 is critical for human natural CD4+CD25+ regulatory T cells to suppress alloimmune response.** *Transplant immunology* 2012, **26:**71-80.

17. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA: **ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28.** *Nature* 1999, **397:**263-266.

18. Harada H, Salama AD, Sho M, Izawa A, Sandner SE, Ito T, Akiba H, Yagita H, Sharpe AH, Freeman GJ, Sayegh MH: **The role of the ICOS-B7h T cell costimulatory pathway in transplantation immunity.** *The Journal of clinical investigation* 2003, **112:**234-243.

19. Hawiger D, Tran E, Du W, Booth CJ, Wen L, Dong C, Flavell RA: **ICOS mediates the development of insulin-dependent diabetes mellitus in nonobese diabetic mice.** *J Immunol* 2008, **180:**3140-3147.

20. Yu D, Tan AH, Hu X, Athanasopoulos V, Simpson N, Silva DG, Hutloff A, Giles KM, Leedman PJ, Lam KP, et al: **Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA.** *Nature* 2007, **450:**299-303.

21. Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Drager R, Eibel H, Fischer B, Schaffer AA, Mages HW, et al: **Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency.** *Nature immunology* 2003, **4:**261-268.

22. Nurieva R, Thomas S, Nguyen T, Martin-Orozco N, Wang Y, Kaja MK, Yu XZ, Dong C: **T-cell tolerance or function is determined by combinatorial costimulatory signals.** *The EMBO journal* 2006, **25:**2623-2633.

23. Mazanet MM, Hughes CC: **B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis.** *J Immunol* 2002, **169:**3581-3588.

24. Saudemont A, Jouy N, Hetuin D, Quesnel B: **NK cells that are activated by CXCL10 can kill dormant tumor cells that resist CTL-mediated lysis and can express B7-H1 that stimulates T cells.** *Blood* 2005, **105:**2428-2435.

25. Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, Pennesi G: **Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway.** *European journal of immunology* 2005, **35:**1482-1490.

26. Hori J, Wang M, Miyashita M, Tanemoto K, Takahashi H, Takemori T, Okumura K, Yagita H, Azuma M: **B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts.** *J Immunol* 2006, **177:**5928-5935.

27. Thompson RH, Kwon ED, Allison JP: **Inhibitors of B7-CD28 costimulation in urologic malignancies.** *Immunotherapy* 2009, **1:**129-139.

28. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, Shin T, Tsuchiya H, Pardoll DM, Okumura K, et al: **Expression of programmed death 1 ligands by murine T cells and APC.** *J Immunol* 2002, **169:**5538-5545.

29. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T: **Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes.** *Int Immunol* 1996, **8:**765-772.

30. Riella LV, Paterson AM, Sharpe AH, Chandraker A: **Role of the PD-1 pathway in the immune response.** *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2012, **12:**2575-2587.

31. Carter L, Fouser LA, Jussif J, Fitz L, Deng B, Wood CR, Collins M, Honjo T, Freeman GJ, Carreno BM: **PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2.** *European journal of immunology* 2002, **32:**634-643.

32. Nishimura H, Honjo T, Minato N: **Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice.** *The Journal of experimental medicine* 2000, **191:**891-898.

33. Whiteside TL: **Inhibiting the inhibitors: evaluating agents targeting cancer immunosuppression.** *Expert opinion on biological therapy* 2010, **10:**1019-1035.

34. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, Koulmanda M, Freeman GJ, Sayegh MH, Sharpe AH: **Tissue expression of PD-L1 mediates peripheral T cell tolerance.** *The Journal of experimental medicine* 2006, **203:**883-895.

35. Dong H, Zhu G, Tamada K, Chen L: **B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion.** *Nature medicine* 1999, **5:**1365-1369.

36. Tseng SY, Otsuji M, Gorski K, Huang X, Slansky JE, Pai SI, Shalabi A, Shin T, Pardoll DM, Tsuchiya H: **B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells.** *The Journal of experimental medicine* 2001, **193:**839-846.

37. Chapoval AI, Ni J, Lau JS, Wilcox RA, Flies DB, Liu D, Dong H, Sica GL, Zhu G, Tamada K, Chen L: **B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production.** *Nature immunology* 2001, **2:**269-274.

38. Sun M, Richards S, Prasad DV, Mai XM, Rudensky A, Dong C: **Characterization of mouse and human B7-H3 genes.** *J Immunol* 2002, **168:**6294-6297.

39. Prasad DV, Nguyen T, Li Z, Yang Y, Duong J, Wang Y, Dong C: **Murine B7-H3 is a negative regulator of T cells.** *J Immunol* 2004, **173:**2500-2506.

40. Suh WK, Gajewska BU, Okada H, Gronski MA, Bertram EM, Dawicki W, Duncan GS, Bukczynski J, Plyte S, Elia A, et al: **The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses.** *Nature immunology* 2003, **4:**899-906.

41. Salceda S, Tang T, Kmet M, Munteanu A, Ghosh M, Macina R, Liu W, Pilkington G, Papkoff J: **The immunomodulatory protein B7-H4 is overexpressed in breast and ovarian cancers and promotes epithelial cell transformation.** *Exp Cell Res* 2005, **306:**128-141.

42. Prasad DV, Richards S, Mai XM, Dong C: **B7S1, a novel B7 family member that negatively regulates T cell activation.** *Immunity* 2003, **18:**863-873.

43. Brandt CS, Baratin M, Yi EC, Kennedy J, Gao Z, Fox B, Haldeman B, Ostrander CD, Kaifu T, Chabannon C, et al: **The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans.** *The Journal of experimental medicine* 2009, **206:**1495-1503.

44. Podojil JR, Miller SD: **Targeting the B7 family of co-stimulatory molecules: successes and challenges.** *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy* 2013, **27:**1-13.

45. Boyden LM, Lewis JM, Barbee SD, Bas A, Girardi M, Hayday AC, Tigelaar RE, Lifton RP: **Skint1, the prototype of a newly identified immunoglobulin superfamily gene cluster, positively selects epidermal gammadelta T cells.** *Nat Genet* 2008, **40:**656-662.

46. Arnett HA, Escobar SS, Viney JL: **Regulation of costimulation in the era of butyrophilins.** *Cytokine* 2009, **46:**370-375.

47. Ogg SL, Weldon AK, Dobbie L, Smith AJ, Mather IH: **Expression of butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets.** *Proc Natl Acad Sci U S A* 2004, **101:**10084-10089.

48. Jeong J, Rao AU, Xu J, Ogg SL, Hathout Y, Fenselau C, Mather IH: **The PRY/SPRY/B30.2 domain of butyrophilin 1A1 (BTN1A1) binds to xanthine oxidoreductase: implications for the function of BTN1A1 in the mammary gland and other tissues.** *J Biol Chem* 2009, **284:**22444-22456.

49. Nguyen T, Liu XK, Zhang Y, Dong C: **BTNL2, a butyrophilin-like molecule that functions to inhibit T cell activation.** *J Immunol* 2006, **176:**7354-7360.

50. Arnett HA, Escobar SS, Gonzalez-Suarez E, Budelsky AL, Steffen LA, Boiani N, Zhang M, Siu G, Brewer AW, Viney JL: **BTNL2, a butyrophilin/B7-like molecule, is a negative costimulatory molecule modulated in intestinal inflammation.** *J Immunol* 2007, **178:**1523-1533.

51. Yamazaki T, Goya I, Graf D, Craig S, Martin-Orozco N, Dong C: **A butyrophilin family member critically inhibits T cell activation.** *J Immunol* 2010, **185:**5907-5914.

52. Bas A, Swamy M, Abeler-Dorner L, Williams G, Pang DJ, Barbee SD, Hayday AC: **Butyrophilin-like 1 encodes an enterocyte protein that selectively regulates functional interactions with T lymphocytes.** *Proc Natl Acad Sci U S A* 2011, **108:**4376-4381.

53. Malcherek G, Mayr L, Roda-Navarro P, Rhodes D, Miller N, Trowsdale J: **The B7 homolog butyrophilin BTN2A1 is a novel ligand for DC-SIGN.** *J Immunol* 2007, **179:**3804-3811.

54. Smith IA, Knezevic BR, Ammann JU, Rhodes DA, Aw D, Palmer DB, Mather IH, Trowsdale J: **BTN1A1, the mammary gland butyrophilin, and BTN2A2 are both inhibitors of T cell activation.** *J Immunol* 2010, **184:**3514-3525.

55. Afrache H, Gouret P, Ainouche S, Pontarotti P, Olive D: **The butyrophilin (BTN) gene family: from milk fat to the regulation of the immune response.** *Immunogenetics* 2012, **64:**781-794.

56. Abeler-Dorner L, Swamy M, Williams G, Hayday AC, Bas A: **Butyrophilins: an emerging family of immune regulators.** *Trends Immunol*, **33:**34-41.

57. Yamazaki T, Goya I, Graf D, Craig S, Martin-Orozco N, Dong C: **A butyrophilin family member critically inhibits T cell activation.** *J Immunol*, **185:**5907-5914.

58. Smith IA, Knezevic BR, Ammann JU, Rhodes DA, Aw D, Palmer DB, Mather IH, Trowsdale J: **BTN1A1, the mammary gland butyrophilin, and BTN2A2 are both inhibitors of T cell activation.** *J Immunol*, **184:**3514-3525.

59. Wijnen PA, Voorter CE, Nelemans PJ, Verschakelen JA, Bekers O, Drent M: **Butyrophilin-like 2 in pulmonary sarcoidosis: a factor for susceptibility and progression?** *Hum Immunol* 2011, **72:**342-347.

60. Scott AP, Laing NG, Mastaglia F, Needham M, Walter MC, Dalakas MC, Allcock RJ: **Recombination mapping of the susceptibility region for sporadic inclusion body myositis within the major histocompatibility complex.** *Journal of neuroimmunology* 2011, **235:**77-83.

61. Mitsunaga S, Hosomichi K, Okudaira Y, Nakaoka H, Kunii N, Suzuki Y, Kuwana M, Sato S, Kaneko Y, Homma Y, et al: **Exome sequencing identifies novel rheumatoid arthritis-susceptible variants in the BTNL2.** *J Hum Genet* 2013, **58:**210-215.

62. Price P, Santoso L, Mastaglia F, Garlepp M, Kok CC, Allcock R, Laing N: **Two major histocompatibility complex haplotypes influence susceptibility to sporadic inclusion body myositis: critical evaluation of an association with HLA-DR3.** *Tissue Antigens* 2004, **64:**575-580.

63. Orozco G, Eerligh P, Sanchez E, Zhernakova S, Roep BO, Gonzalez-Gay MA, Lopez-Nevot MA, Callejas JL, Hidalgo C, Pascual-Salcedo D, et al: **Analysis of a functional BTNL2 polymorphism in type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus.** *Hum Immunol* 2005, **66:**1235-1241.

64. Johnson CM, Traherne JA, Jamieson SE, Tremelling M, Bingham S, Parkes M, Blackwell JM, Trowsdale J: **Analysis of the BTNL2 truncating splice site mutation in tuberculosis, leprosy and Crohn's disease.** *Tissue Antigens* 2007, **69:**236-241.

65. Konno S, Takahashi D, Hizawa N, Hattori T, Takahashi A, Isada A, Maeda Y, Huang SK, Nishimura M: **Genetic impact of a butyrophilin-like 2 (BTNL2) gene variation on specific IgE responsiveness to Dermatophagoides farinae (Der f) in Japanese.** *Allergol Int* 2009, **58:**29-35.

66. Compte E, Pontarotti P, Collette Y, Lopez M, Olive D: **Frontline: Characterization of BT3 molecules belonging to the B7 family expressed on immune cells.** *European journal of immunology* 2004, **34:**2089-2099.

67. Yamashiro H, Yoshizaki S, Tadaki T, Egawa K, Seo N: **Stimulation of human butyrophilin 3 molecules results in negative regulation of cellular immunity.** *J Leukoc Biol* 2010, **88:**757-767.

68. Messal N, Mamessier E, Sylvain A, Celis-Gutierrez J, Thibult ML, Chetaille B, Firaguay G, Pastor S, Guillaume Y, Wang Q, et al: **Differential role for CD277 as a co-regulator of the immune signal in T and NK cells.** *European journal of immunology* 2011, **41:**3443-3454.

69. Harly C, Guillaume Y, Nedellec S, Peigne CM, Monkkonen H, Monkkonen J, Li J, Kuball J, Adams EJ, Netzer S, et al: **Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human gammadelta T-cell subset.** *Blood* 2012, **120:**2269-2279.

70. Palakodeti A, Sandstrom A, Sundaresan L, Harly C, Nedellec S, Olive D, Scotet E, Bonneville M, Adams EJ: **The molecular basis for modulation of human Vgamma9Vdelta2 T cell responses by CD277/butyrophilin-3 (BTN3A)-specific antibodies.** *J Biol Chem* 2012, **287:**32780-32790.

71. Grivennikov SI, Greten FR, Karin M: **Immunity, inflammation, and cancer.** *Cell* 2010, **140:**883-899.

72. Pardoll DM: **The blockade of immune checkpoints in cancer immunotherapy.** *Nature reviews Cancer* 2012, **12:**252-264.

73. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, et al: **Improved survival with ipilimumab in patients with metastatic melanoma.** *N Engl J Med* 2010, **363:**711-723.

74. Keir ME, Butte MJ, Freeman GJ, Sharpe AH: **PD-1 and its ligands in tolerance and immunity.** *Annual review of immunology* 2008, **26:**677-704.

75. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, et al: **Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion.** *Nature medicine* 2002, **8:**793-800.

76. Zou W, Chen L: **Inhibitory B7-family molecules in the tumour microenvironment.** *Nature reviews Immunology* 2008, **8:**467-477.

77. Ghebeh H, Tulbah A, Mohammed S, Elkum N, Bin Amer SM, Al-Tweigeri T, Dermime S: **Expression of B7-H1 in breast cancer patients is strongly associated with high proliferative Ki-67-expressing tumor cells.** *International journal of cancer Journal international du cancer* 2007, **121:**751-758.

78. Watanabe M, Takagi Y, Kotani M, Hara Y, Inamine A, Hayashi K, Ogawa S, Takeda K, Tanabe K, Abe R: **Down-regulation of ICOS ligand by interaction with ICOS functions as a regulatory mechanism for immune responses.** *J Immunol* 2008, **180:**5222-5234.

79. Aplan PD: **Causes of oncogenic chromosomal translocation.** *Trends in genetics : TIG* 2006, **22:**46-55.

80. Gordon MS, Kato RM, Lansigan F, Thompson AA, Wall R, Rawlings DJ: **Aberrant B cell receptor signaling from B29 (Igbeta, CD79b) gene mutations of chronic lymphocytic leukemia B cells.** *Proc Natl Acad Sci U S A* 2000, **97:**5504-5509.

81. Vogelstein B, Kinzler KW: **The multistep nature of cancer.** *Trends in genetics : TIG* 1993, **9:**138-141.

82. Rabbitts TH: **Chromosomal translocations in human cancer.** *Nature* 1994, **372:**143-149.

83. Difilippantonio MJ, Petersen S, Chen HT, Johnson R, Jasin M, Kanaar R, Ried T, Nussenzweig A: **Evidence for replicative repair of DNA double-strand breaks leading to oncogenic translocation and gene amplification.** *The Journal of experimental medicine* 2002, **196:**469-480.

84. Pui CH, Carroll WL, Meshinchi S, Arceci RJ: **Biology, risk stratification, and therapy of pediatric acute leukemias: an update.** *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2011, **29:**551-565.

85. Rubnitz JE, Crist WM: **Molecular genetics of childhood cancer: implications for pathogenesis, diagnosis, and treatment.** *Pediatrics* 1997, **100:**101-108.

86. Pieters R, Carroll WL: **Biology and treatment of acute lymphoblastic leukemia.** *Hematology/oncology clinics of North America* 2010, **24:**1-18.

87. Mullighan CG: **The molecular genetic makeup of acute lymphoblastic leukemia.** *Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program* 2012, **2012:**389-396.

88. Ibagy A, Silva DB, Seiben J, Winneshoffer AP, Costa TE, Dacoregio JS, Costa I, Faraco D: **Acute lymphoblastic leukemia in infants: 20 years of experience.** *Jornal de pediatria* 2013, **89:**64-69.

89. Inaba H, Greaves M, Mullighan CG: **Acute lymphoblastic leukaemia.** *Lancet* 2013, **381:**1943-1955.

90. Rhen T, Cidlowski JA: **Antiinflammatory action of glucocorticoids--new mechanisms for old drugs.** *N Engl J Med* 2005, **353:**1711-1723.

91. Alexanian R, Dimopoulos M: **The treatment of multiple myeloma.** *N Engl J Med* 1994, **330:**484-489.

92. Gaynon PS, Carrel AL: **Glucocorticosteroid therapy in childhood acute lymphoblastic leukemia.** *Advances in experimental medicine and biology* 1999, **457:**593-605.

93. Moalli PA, Rosen ST: **Glucocorticoid receptors and resistance to glucocorticoids in hematologic malignancies.** *Leukemia & lymphoma* 1994, **15:**363-374.

94. Frankfurt O, Rosen ST: **Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates.** *Current opinion in oncology* 2004, **16:**553-563.

95. Newton R, Holden NS: **Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor?** *Molecular pharmacology* 2007, **72:**799-809.

96. Barnes PJ: **Glucocorticosteroids: current and future directions.** *British journal of pharmacology* 2011, **163:**29-43.

97. Baschant U, Tuckermann J: **The role of the glucocorticoid receptor in inflammation and immunity.** *The Journal of steroid biochemistry and molecular biology* 2010, **120:**69-75.

98. Wang JC, Gray NE, Kuo T, Harris CA: **Regulation of triglyceride metabolism by glucocorticoid receptor.** *Cell & bioscience* 2012, **2:**19.

99. Rogatsky I, Trowbridge JM, Garabedian MJ: **Glucocorticoid receptor-mediated cell cycle arrest is achieved through distinct cell-specific transcriptional regulatory mechanisms.** *Molecular and cellular biology* 1997, **17:**3181-3193.

100. Reichardt HM, Tronche F, Berger S, Kellendonk C, Schutz G: **New insights into glucocorticoid and mineralocorticoid signaling: lessons from gene targeting.** *Adv Pharmacol* 2000, **47:**1-21.

101. Pazirandeh A, Xue Y, Prestegaard T, Jondal M, Okret S: **Effects of altered glucocorticoid sensitivity in the T cell lineage on thymocyte and T cell homeostasis.** *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2002, **16:**727-729.

102. Kofler R, Schmidt S, Kofler A, Ausserlechner MJ: **Resistance to glucocorticoid-induced apoptosis in lymphoblastic leukemia.** *The Journal of endocrinology* 2003, **178:**19-27.

103. Ramdas L, Cogdell DE, Jia JY, Taylor EE, Dunmire VR, Hu L, Hamilton SR, Zhang W: **Improving signal intensities for genes with low-expression on oligonucleotide microarrays.** *BMC Genomics* 2004, **5:**35.

104. McConkey DJ, Chandra J: **Protease activation and glucocorticoid-induced apoptosis in chronic lymphocytic leukemia.** *Leukemia & lymphoma* 1999, **33:**421-431.

105. Inaba H, Pui CH: **Glucocorticoid use in acute lymphoblastic leukaemia.** *The lancet oncology* 2010, **11:**1096-1106.

106. Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS, Elia A, de la Pompa JL, Kagi D, Khoo W, et al: **Differential requirement for caspase 9 in apoptotic pathways in vivo.** *Cell* 1998, **94:**339-352.

107. Yoshino T, Asada H, Ando Y, Fujii H, Yamaguchi Y, Yoshikawa K, Itami S: **Impaired responses of peripheral blood mononuclear cells to T-cell stimulants in alopecia areata patients with a poor response to topical immunotherapy.** *The British journal of dermatology* 2001, **145:**415-421.

108. Thornberry NA: **Caspases: key mediators of apoptosis.** *Chemistry & biology* 1998, **5:**R97-103.

109. Bellosillo B, Dalmau M, Colomer D, Gil J: **Involvement of CED-3/ICE proteases in the apoptosis of B-chronic lymphocytic leukemia cells.** *Blood* 1997, **89:**3378-3384.

110. Sionov RV: **MicroRNAs and Glucocorticoid-Induced Apoptosis in Lymphoid Malignancies.** *ISRN hematology* 2013, **2013:**348212.

111. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM, Strasser A: **Proapoptotic Bcl-2 relative Bim required for certain**

**apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity.** *Science* 1999, **286:**1735-1738.

112. Nuutinen U, Ropponen A, Suoranta S, Eeva J, Eray M, Pellinen R, Wahlfors J, Pelkonen J: **Dexamethasone-induced apoptosis and up-regulation of Bim is dependent on glycogen synthase kinase-3.** *Leukemia research* 2009, **33:**1714-1717.

113. Kim H, Tu HC, Ren D, Takeuchi O, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH: **Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis.** *Molecular cell* 2009, **36:**487-499.

114. Wang S, Lim G, Zeng Q, Sung B, Ai Y, Guo G, Yang L, Mao J: **Expression of central glucocorticoid receptors after peripheral nerve injury contributes to neuropathic pain behaviors in rats.** *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2004, **24:**8595-8605.

115. Delfino DV, Agostini M, Spinicelli S, Vito P, Riccardi C: **Decrease of Bcl-xL and augmentation of thymocyte apoptosis in GILZ overexpressing transgenic mice.** *Blood* 2004, **104:**4134-4141.

116. Ayroldi E, Zollo O, Bastianelli A, Marchetti C, Agostini M, Di Virgilio R, Riccardi C: **GILZ mediates the antiproliferative activity of glucocorticoids by negative regulation of Ras signaling.** *The Journal of clinical investigation* 2007, **117:**1605-1615.

117. Wang Z, Malone MH, Thomenius MJ, Zhong F, Xu F, Distelhorst CW: **Dexamethasone-induced gene 2 (dig2) is a novel pro-survival stress gene induced rapidly by diverse apoptotic signals.** *J Biol Chem* 2003, **278:**27053-27058.

118. Radu CG, Cheng D, Nijagal A, Riedinger M, McLaughlin J, Yang LV, Johnson J, Witte ON: **Normal immune development and glucocorticoid-induced thymocyte apoptosis in mice deficient for the T-cell death-associated gene 8 receptor.** *Molecular and cellular biology* 2006, **26:**668-677.

119. Schrappe M, Reiter A, Ludwig WD, Harbott J, Zimmermann M, Hiddemann W, Niemeyer C, Henze G, Feldges A, Zintl F, et al: **Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group.** *Blood* 2000, **95:**3310-3322.

120. Dordelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, Gadner H, Riehm H, Schrappe M: **Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia.** *Blood* 1999, **94:**1209-1217.

121. Zwaan CM, Kaspers GJ, Pieters R, Ramakers-Van Woerden NL, den Boer ML, Wunsche R, Rottier MM, Hahlen K, van Wering ER, Janka-Schaub GE, et al:

**Cellular drug resistance profiles in childhood acute myeloid leukemia: differences between FAB types and comparison with acute lymphoblastic leukemia.** *Blood* 2000, **96:**2879-2886.

1. Ceeraz S, Nowak EC, Noelle RJ: **B7 family checkpoint regulators in immune regulation and disease.** *Trends in immunology* 2013.

2. Greenfield EA, Nguyen KA, Kuchroo VK: **CD28/B7 costimulation: a review.** *Critical reviews in immunology* 1998, **18:**389-418.

3. Rulifson IC, Sperling AI, Fields PE, Fitch FW, Bluestone JA: **CD28 costimulation promotes the production of Th2 cytokines.** *J Immunol* 1997, **158:**658-665.

4. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, Kawai K, Ohashi PS, Thompson CB, Mak TW: **Differential T cell costimulatory requirements in CD28-deficient mice.** *Science* 1993, **261:**609-612.

5. Seliger B, Quandt D: **The expression, function, and clinical relevance of B7 family members in cancer.** *Cancer immunology, immunotherapy : CII* 2012, **61:**1327-1341.

6. Flies DB, Chen L: **The new B7s: playing a pivotal role in tumor immunity.** *J Immunother* 2007, **30:**251-260.

7. Collins M, Ling V, Carreno BM: **The B7 family of immune-regulatory ligands.** *Genome Biol* 2005, **6:**223.

8. Greaves P, Gribben JG: **The role of B7 family molecules in hematologic malignancy.** *Blood* 2013, **121:**734-744.

9. Acuto O, Mise-Omata S, Mangino G, Michel F: **Molecular modifiers of T cell antigen receptor triggering threshold: the mechanism of CD28 costimulatory receptor.** *Immunological reviews* 2003, **192:**21-31.

10. Chambers CA, Kuhns MS, Egen JG, Allison JP: **CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy.** *Annual review of immunology* 2001, **19:**565-594.

11. Seliger B, Marincola FM, Ferrone S, Abken H: **The complex role of B7 molecules in tumor immunology.** *Trends in molecular medicine* 2008, **14:**550-559.

12. Chambers CA, Allison JP: **Costimulatory regulation of T cell function.** *Current opinion in cell biology* 1999, **11:**203-210.

13. Greenwald RJ, Freeman GJ, Sharpe AH: **The B7 family revisited.** *Annual review of immunology* 2005, **23:**515-548.

14. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S: **CTLA-4 control over Foxp3+ regulatory T cell function.** *Science* 2008, **322:**271-275.

15. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP: **Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies.** *The Journal of experimental medicine* 2009, **206:**1717-1725.

16. Sun L, Wu J, Yi S: **Foxp3 is critical for human natural CD4+CD25+ regulatory T cells to suppress alloimmune response.** *Transplant immunology* 2012, **26:**71-80.

17. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA: **ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28.** *Nature* 1999, **397:**263-266.

18. Harada H, Salama AD, Sho M, Izawa A, Sandner SE, Ito T, Akiba H, Yagita H, Sharpe AH, Freeman GJ, Sayegh MH: **The role of the ICOS-B7h T cell costimulatory pathway in transplantation immunity.** *The Journal of clinical investigation* 2003, **112:**234-243.

19. Hawiger D, Tran E, Du W, Booth CJ, Wen L, Dong C, Flavell RA: **ICOS mediates the development of insulin-dependent diabetes mellitus in nonobese diabetic mice.** *J Immunol* 2008, **180:**3140-3147.

20. Yu D, Tan AH, Hu X, Athanasopoulos V, Simpson N, Silva DG, Hutloff A, Giles KM, Leedman PJ, Lam KP, et al: **Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA.** *Nature* 2007, **450:**299-303.

21. Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Drager R, Eibel H, Fischer B, Schaffer AA, Mages HW, et al: **Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency.** *Nature immunology* 2003, **4:**261-268.

22. Nurieva R, Thomas S, Nguyen T, Martin-Orozco N, Wang Y, Kaja MK, Yu XZ, Dong C: **T-cell tolerance or function is determined by combinatorial costimulatory signals.** *The EMBO journal* 2006, **25:**2623-2633.

23. Mazanet MM, Hughes CC: **B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis.** *J Immunol* 2002, **169:**3581-3588.

24. Saudemont A, Jouy N, Hetuin D, Quesnel B: **NK cells that are activated by CXCL10 can kill dormant tumor cells that resist CTL-mediated lysis and can express B7-H1 that stimulates T cells.** *Blood* 2005, **105:**2428-2435.

25. Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, Pennesi G: **Bone marrow mesenchymal progenitor cells inhibit lymphocyte**

**proliferation by activation of the programmed death 1 pathway.** *European journal of immunology* 2005, **35:**1482-1490.

26. Hori J, Wang M, Miyashita M, Tanemoto K, Takahashi H, Takemori T, Okumura K, Yagita H, Azuma M: **B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts.** *J Immunol* 2006, **177:**5928-5935.

27. Thompson RH, Kwon ED, Allison JP: **Inhibitors of B7-CD28 costimulation in urologic malignancies.** *Immunotherapy* 2009, **1:**129-139.

28. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, Shin T, Tsuchiya H, Pardoll DM, Okumura K, et al: **Expression of programmed death 1 ligands by murine T cells and APC.** *J Immunol* 2002, **169:**5538-5545.

29. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T: **Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes.** *Int Immunol* 1996, **8:**765-772.

30. Riella LV, Paterson AM, Sharpe AH, Chandraker A: **Role of the PD-1 pathway in the immune response.** *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2012, **12:**2575-2587.

31. Carter L, Fouser LA, Jussif J, Fitz L, Deng B, Wood CR, Collins M, Honjo T, Freeman GJ, Carreno BM: **PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2.** *European journal of immunology* 2002, **32:**634-643.

32. Nishimura H, Honjo T, Minato N: **Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice.** *The Journal of experimental medicine* 2000, **191:**891-898.

33. Whiteside TL: **Inhibiting the inhibitors: evaluating agents targeting cancer immunosuppression.** *Expert opinion on biological therapy* 2010, **10:**1019-1035.

34. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, Koulmanda M, Freeman GJ, Sayegh MH, Sharpe AH: **Tissue expression of PD-L1 mediates peripheral T cell tolerance.** *The Journal of experimental medicine* 2006, **203:**883-895.

35. Dong H, Zhu G, Tamada K, Chen L: **B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion.** *Nature medicine* 1999, **5:**1365-1369.

36. Tseng SY, Otsuji M, Gorski K, Huang X, Slansky JE, Pai SI, Shalabi A, Shin T, Pardoll DM, Tsuchiya H: **B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells.** *The Journal of experimental medicine* 2001, **193:**839-846.

37. Chapoval AI, Ni J, Lau JS, Wilcox RA, Flies DB, Liu D, Dong H, Sica GL, Zhu G, Tamada K, Chen L: **B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production.** *Nature immunology* 2001, **2:**269-274.

38. Sun M, Richards S, Prasad DV, Mai XM, Rudensky A, Dong C: **Characterization of mouse and human B7-H3 genes.** *J Immunol* 2002, **168:**6294-6297.

39. Prasad DV, Nguyen T, Li Z, Yang Y, Duong J, Wang Y, Dong C: **Murine B7-H3 is a negative regulator of T cells.** *J Immunol* 2004, **173:**2500-2506.

40. Suh WK, Gajewska BU, Okada H, Gronski MA, Bertram EM, Dawicki W, Duncan GS, Bukczynski J, Plyte S, Elia A, et al: **The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses.** *Nature immunology* 2003, **4:**899-906.

41. Salceda S, Tang T, Kmet M, Munteanu A, Ghosh M, Macina R, Liu W, Pilkington G, Papkoff J: **The immunomodulatory protein B7-H4 is overexpressed in breast and ovarian cancers and promotes epithelial cell transformation.** *Exp Cell Res* 2005, **306:**128-141.

42. Prasad DV, Richards S, Mai XM, Dong C: **B7S1, a novel B7 family member that negatively regulates T cell activation.** *Immunity* 2003, **18:**863-873.

43. Brandt CS, Baratin M, Yi EC, Kennedy J, Gao Z, Fox B, Haldeman B, Ostrander CD, Kaifu T, Chabannon C, et al: **The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans.** *The Journal of experimental medicine* 2009, **206:**1495-1503.

44. Podojil JR, Miller SD: **Targeting the B7 family of co-stimulatory molecules: successes and challenges.** *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy* 2013, **27:**1-13.

45. Boyden LM, Lewis JM, Barbee SD, Bas A, Girardi M, Hayday AC, Tigelaar RE, Lifton RP: **Skint1, the prototype of a newly identified immunoglobulin superfamily gene cluster, positively selects epidermal gammadelta T cells.** *Nat Genet* 2008, **40:**656-662.

46. Arnett HA, Escobar SS, Viney JL: **Regulation of costimulation in the era of butyrophilins.** *Cytokine* 2009, **46:**370-375.

47. Ogg SL, Weldon AK, Dobbie L, Smith AJ, Mather IH: **Expression of butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets.** *Proc Natl Acad Sci U S A* 2004, **101:**10084-10089.

48. Jeong J, Rao AU, Xu J, Ogg SL, Hathout Y, Fenselau C, Mather IH: **The PRY/SPRY/B30.2 domain of butyrophilin 1A1 (BTN1A1) binds to xanthine oxidoreductase: implications for the function of BTN1A1 in the mammary gland and other tissues.** *J Biol Chem* 2009, **284:**22444-22456.

49. Nguyen T, Liu XK, Zhang Y, Dong C: **BTNL2, a butyrophilin-like molecule that functions to inhibit T cell activation.** *J Immunol* 2006, **176:**7354-7360.

50. Arnett HA, Escobar SS, Gonzalez-Suarez E, Budelsky AL, Steffen LA, Boiani N, Zhang M, Siu G, Brewer AW, Viney JL: **BTNL2, a butyrophilin/B7-like molecule, is a negative costimulatory molecule modulated in intestinal inflammation.** *J Immunol* 2007, **178:**1523-1533.

51. Yamazaki T, Goya I, Graf D, Craig S, Martin-Orozco N, Dong C: **A butyrophilin family member critically inhibits T cell activation.** *J Immunol* 2010, **185:**5907-5914.

52. Bas A, Swamy M, Abeler-Dorner L, Williams G, Pang DJ, Barbee SD, Hayday AC: **Butyrophilin-like 1 encodes an enterocyte protein that selectively regulates functional interactions with T lymphocytes.** *Proc Natl Acad Sci U S A* 2011, **108:**4376-4381.

53. Malcherek G, Mayr L, Roda-Navarro P, Rhodes D, Miller N, Trowsdale J: **The B7 homolog butyrophilin BTN2A1 is a novel ligand for DC-SIGN.** *J Immunol* 2007, **179:**3804-3811.

54. Smith IA, Knezevic BR, Ammann JU, Rhodes DA, Aw D, Palmer DB, Mather IH, Trowsdale J: **BTN1A1, the mammary gland butyrophilin, and BTN2A2 are both inhibitors of T cell activation.** *J Immunol* 2010, **184:**3514-3525.

55. Afrache H, Gouret P, Ainouche S, Pontarotti P, Olive D: **The butyrophilin (BTN) gene family: from milk fat to the regulation of the immune response.** *Immunogenetics* 2012, **64:**781-794.

56. Abeler-Dorner L, Swamy M, Williams G, Hayday AC, Bas A: **Butyrophilins: an emerging family of immune regulators.** *Trends Immunol*, **33:**34-41.

57. Yamazaki T, Goya I, Graf D, Craig S, Martin-Orozco N, Dong C: **A butyrophilin family member critically inhibits T cell activation.** *J Immunol*, **185:**5907-5914.

58. Smith IA, Knezevic BR, Ammann JU, Rhodes DA, Aw D, Palmer DB, Mather IH, Trowsdale J: **BTN1A1, the mammary gland butyrophilin, and BTN2A2 are both inhibitors of T cell activation.** *J Immunol*, **184:**3514-3525.

59. Wijnen PA, Voorter CE, Nelemans PJ, Verschakelen JA, Bekers O, Drent M: **Butyrophilin-like 2 in pulmonary sarcoidosis: a factor for susceptibility and progression?** *Hum Immunol* 2011, **72:**342-347.

60. Scott AP, Laing NG, Mastaglia F, Needham M, Walter MC, Dalakas MC, Allcock RJ: **Recombination mapping of the susceptibility region for sporadic inclusion body myositis within the major histocompatibility complex.** *Journal of neuroimmunology* 2011, **235:**77-83.

61. Mitsunaga S, Hosomichi K, Okudaira Y, Nakaoka H, Kunii N, Suzuki Y, Kuwana M, Sato S, Kaneko Y, Homma Y, et al: **Exome sequencing identifies novel rheumatoid arthritis-susceptible variants in the BTNL2.** *J Hum Genet* 2013, **58:**210-215.

62. Price P, Santoso L, Mastaglia F, Garlepp M, Kok CC, Allcock R, Laing N: **Two major histocompatibility complex haplotypes influence susceptibility to sporadic inclusion body myositis: critical evaluation of an association with HLA-DR3.** *Tissue Antigens* 2004, **64:**575-580.

63. Orozco G, Eerligh P, Sanchez E, Zhernakova S, Roep BO, Gonzalez-Gay MA, Lopez-Nevot MA, Callejas JL, Hidalgo C, Pascual-Salcedo D, et al: **Analysis of a functional BTNL2 polymorphism in type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus.** *Hum Immunol* 2005, **66:**1235-1241.

64. Johnson CM, Traherne JA, Jamieson SE, Tremelling M, Bingham S, Parkes M, Blackwell JM, Trowsdale J: **Analysis of the BTNL2 truncating splice site mutation in tuberculosis, leprosy and Crohn's disease.** *Tissue Antigens* 2007, **69:**236-241.

65. Konno S, Takahashi D, Hizawa N, Hattori T, Takahashi A, Isada A, Maeda Y, Huang SK, Nishimura M: **Genetic impact of a butyrophilin-like 2 (BTNL2) gene variation on specific IgE responsiveness to Dermatophagoides farinae (Der f) in Japanese.** *Allergol Int* 2009, **58:**29-35.

66. Compte E, Pontarotti P, Collette Y, Lopez M, Olive D: **Frontline: Characterization of BT3 molecules belonging to the B7 family expressed on immune cells.** *European journal of immunology* 2004, **34:**2089-2099.

67. Yamashiro H, Yoshizaki S, Tadaki T, Egawa K, Seo N: **Stimulation of human butyrophilin 3 molecules results in negative regulation of cellular immunity.** *J Leukoc Biol* 2010, **88:**757-767.

68. Messal N, Mamessier E, Sylvain A, Celis-Gutierrez J, Thibult ML, Chetaille B, Firaguay G, Pastor S, Guillaume Y, Wang Q, et al: **Differential role for CD277 as a co-regulator of the immune signal in T and NK cells.** *European journal of immunology* 2011, **41:**3443-3454.

69. Harly C, Guillaume Y, Nedellec S, Peigne CM, Monkkonen H, Monkkonen J, Li J, Kuball J, Adams EJ, Netzer S, et al: **Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human gammadelta T-cell subset.** *Blood* 2012, **120:**2269-2279.

70. Palakodeti A, Sandstrom A, Sundaresan L, Harly C, Nedellec S, Olive D, Scotet E, Bonneville M, Adams EJ: **The molecular basis for modulation of human Vgamma9Vdelta2 T cell responses by CD277/butyrophilin-3 (BTN3A)-specific antibodies.** *J Biol Chem* 2012, **287:**32780-32790.

71. Grivennikov SI, Greten FR, Karin M: **Immunity, inflammation, and cancer.** *Cell* 2010, **140:**883-899.

72. Pardoll DM: **The blockade of immune checkpoints in cancer immunotherapy.** *Nature reviews Cancer* 2012, **12:**252-264.

73. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, et al: **Improved survival with ipilimumab in patients with metastatic melanoma.** *N Engl J Med* 2010, **363:**711-723.

74. Keir ME, Butte MJ, Freeman GJ, Sharpe AH: **PD-1 and its ligands in tolerance and immunity.** *Annual review of immunology* 2008, **26:**677-704.

75. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, et al: **Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion.** *Nature medicine* 2002, **8:**793-800.

76. Zou W, Chen L: **Inhibitory B7-family molecules in the tumour microenvironment.** *Nature reviews Immunology* 2008, **8:**467-477.

77. Ghebeh H, Tulbah A, Mohammed S, Elkum N, Bin Amer SM, Al-Tweigeri T, Dermime S: **Expression of B7-H1 in breast cancer patients is strongly associated with high proliferative Ki-67-expressing tumor cells.** *International journal of cancer Journal international du cancer* 2007, **121:**751-758.

78. Watanabe M, Takagi Y, Kotani M, Hara Y, Inamine A, Hayashi K, Ogawa S, Takeda K, Tanabe K, Abe R: **Down-regulation of ICOS ligand by interaction with ICOS functions as a regulatory mechanism for immune responses.** *J Immunol* 2008, **180:**5222-5234.

79. Aplan PD: **Causes of oncogenic chromosomal translocation.** *Trends in genetics : TIG* 2006, **22:**46-55.

80. Gordon MS, Kato RM, Lansigan F, Thompson AA, Wall R, Rawlings DJ: **Aberrant B cell receptor signaling from B29 (Igbeta, CD79b) gene mutations of chronic lymphocytic leukemia B cells.** *Proc Natl Acad Sci U S A* 2000, **97:**5504-5509.

81. Vogelstein B, Kinzler KW: **The multistep nature of cancer.** *Trends in genetics : TIG* 1993, **9:**138-141.

82. Rabbitts TH: **Chromosomal translocations in human cancer.** *Nature* 1994, **372:**143-149.

83. Difilippantonio MJ, Petersen S, Chen HT, Johnson R, Jasin M, Kanaar R, Ried T, Nussenzweig A: **Evidence for replicative repair of DNA double-strand breaks leading to oncogenic translocation and gene amplification.** *The Journal of experimental medicine* 2002, **196:**469-480.

84. Pui CH, Carroll WL, Meshinchi S, Arceci RJ: **Biology, risk stratification, and therapy of pediatric acute leukemias: an update.** *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2011, **29:**551-565.

85. Rubnitz JE, Crist WM: **Molecular genetics of childhood cancer: implications for pathogenesis, diagnosis, and treatment.** *Pediatrics* 1997, **100:**101-108.

86. Pieters R, Carroll WL: **Biology and treatment of acute lymphoblastic leukemia.** *Hematology/oncology clinics of North America* 2010, **24:**1-18.

87. Mullighan CG: **The molecular genetic makeup of acute lymphoblastic leukemia.** *Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program* 2012, **2012:**389-396.

88. Ibagy A, Silva DB, Seiben J, Winneshoffer AP, Costa TE, Dacoregio JS, Costa I, Faraco D: **Acute lymphoblastic leukemia in infants: 20 years of experience.** *Jornal de pediatria* 2013, **89:**64-69.

89. Inaba H, Greaves M, Mullighan CG: **Acute lymphoblastic leukaemia.** *Lancet* 2013, **381:**1943-1955.

90. Rhen T, Cidlowski JA: **Antiinflammatory action of glucocorticoids--new mechanisms for old drugs.** *N Engl J Med* 2005, **353:**1711-1723.

91. Alexanian R, Dimopoulos M: **The treatment of multiple myeloma.** *N Engl J Med* 1994, **330:**484-489.

92. Gaynon PS, Carrel AL: **Glucocorticosteroid therapy in childhood acute lymphoblastic leukemia.** *Advances in experimental medicine and biology* 1999, **457:**593-605.

93. Moalli PA, Rosen ST: **Glucocorticoid receptors and resistance to glucocorticoids in hematologic malignancies.** *Leukemia & lymphoma* 1994, **15:**363-374.

94. Frankfurt O, Rosen ST: **Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates.** *Current opinion in oncology* 2004, **16:**553-563.

95. Newton R, Holden NS: **Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor?** *Molecular pharmacology* 2007, **72:**799-809.

96. Barnes PJ: **Glucocorticosteroids: current and future directions.** *British journal of pharmacology* 2011, **163:**29-43.

97. Baschant U, Tuckermann J: **The role of the glucocorticoid receptor in inflammation and immunity.** *The Journal of steroid biochemistry and molecular biology* 2010, **120:**69-75.

98. Wang JC, Gray NE, Kuo T, Harris CA: **Regulation of triglyceride metabolism by glucocorticoid receptor.** *Cell & bioscience* 2012, **2:**19.

99. Rogatsky I, Trowbridge JM, Garabedian MJ: **Glucocorticoid receptor-mediated cell cycle arrest is achieved through distinct cell-specific transcriptional regulatory mechanisms.** *Molecular and cellular biology* 1997, **17:**3181-3193.

100. Reichardt HM, Tronche F, Berger S, Kellendonk C, Schutz G: **New insights into glucocorticoid and mineralocorticoid signaling: lessons from gene targeting.** *Adv Pharmacol* 2000, **47:**1-21.

101. Pazirandeh A, Xue Y, Prestegaard T, Jondal M, Okret S: **Effects of altered glucocorticoid sensitivity in the T cell lineage on thymocyte and T cell homeostasis.** *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2002, **16:**727-729.

102. Kofler R, Schmidt S, Kofler A, Ausserlechner MJ: **Resistance to glucocorticoid-induced apoptosis in lymphoblastic leukemia.** *The Journal of endocrinology* 2003, **178:**19-27.

103. Ramdas L, Cogdell DE, Jia JY, Taylor EE, Dunmire VR, Hu L, Hamilton SR, Zhang W: **Improving signal intensities for genes with low-expression on oligonucleotide microarrays.** *BMC Genomics* 2004, **5:**35.

104. McConkey DJ, Chandra J: **Protease activation and glucocorticoid-induced apoptosis in chronic lymphocytic leukemia.** *Leukemia & lymphoma* 1999, **33:**421-431.

105. Inaba H, Pui CH: **Glucocorticoid use in acute lymphoblastic leukaemia.** *The lancet oncology* 2010, **11:**1096-1106.

106. Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS, Elia A, de la Pompa JL, Kagi D, Khoo W, et al: **Differential requirement for caspase 9 in apoptotic pathways in vivo.** *Cell* 1998, **94:**339-352.

107. Yoshino T, Asada H, Ando Y, Fujii H, Yamaguchi Y, Yoshikawa K, Itami S: **Impaired responses of peripheral blood mononuclear cells to T-cell stimulants in alopecia areata patients with a poor response to topical immunotherapy.** *The British journal of dermatology* 2001, **145:**415-421.

108. Thornberry NA: **Caspases: key mediators of apoptosis.** *Chemistry & biology* 1998, **5:**R97-103.

109. Bellosillo B, Dalmau M, Colomer D, Gil J: **Involvement of CED-3/ICE proteases in the apoptosis of B-chronic lymphocytic leukemia cells.** *Blood* 1997, **89:**3378-3384.

110. Sionov RV: **MicroRNAs and Glucocorticoid-Induced Apoptosis in Lymphoid Malignancies.** *ISRN hematology* 2013, **2013:**348212.

111. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM, Strasser A: **Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity.** *Science* 1999, **286:**1735-1738.

112. Nuutinen U, Ropponen A, Suoranta S, Eeva J, Eray M, Pellinen R, Wahlfors J, Pelkonen J: **Dexamethasone-induced apoptosis and up-regulation of Bim is dependent on glycogen synthase kinase-3.** *Leukemia research* 2009, **33:**1714-1717.

113. Kim H, Tu HC, Ren D, Takeuchi O, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH: **Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis.** *Molecular cell* 2009, **36:**487-499.

114. Wang S, Lim G, Zeng Q, Sung B, Ai Y, Guo G, Yang L, Mao J: **Expression of central glucocorticoid receptors after peripheral nerve injury contributes to neuropathic pain behaviors in rats.** *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2004, **24:**8595-8605.

115. Delfino DV, Agostini M, Spinicelli S, Vito P, Riccardi C: **Decrease of Bcl-xL and augmentation of thymocyte apoptosis in GILZ overexpressing transgenic mice.** *Blood* 2004, **104:**4134-4141.

116. Ayroldi E, Zollo O, Bastianelli A, Marchetti C, Agostini M, Di Virgilio R, Riccardi C: **GILZ mediates the antiproliferative activity of glucocorticoids by negative regulation of Ras signaling.** *The Journal of clinical investigation* 2007, **117:**1605-1615.

117. Wang Z, Malone MH, Thomenius MJ, Zhong F, Xu F, Distelhorst CW: **Dexamethasone-induced gene 2 (dig2) is a novel pro-survival stress gene induced rapidly by diverse apoptotic signals.** *J Biol Chem* 2003, **278:**27053-27058.

118. Radu CG, Cheng D, Nijagal A, Riedinger M, McLaughlin J, Yang LV, Johnson J, Witte ON: **Normal immune development and glucocorticoid-induced thymocyte apoptosis in mice deficient for the T-cell death-associated gene 8 receptor.** *Molecular and cellular biology* 2006, **26:**668-677.

119. Schrappe M, Reiter A, Ludwig WD, Harbott J, Zimmermann M, Hiddemann W, Niemeyer C, Henze G, Feldges A, Zintl F, et al: **Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90.**

**German-Austrian-Swiss ALL-BFM Study Group.** *Blood* 2000, **95:**3310-3322.

120. Dordelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, Gadner H, Riehm H, Schrappe M: **Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia.** *Blood* 1999, **94:**1209-1217.

121. Zwaan CM, Kaspers GJ, Pieters R, Ramakers-Van Woerden NL, den Boer ML, Wunsche R, Rottier MM, Hahlen K, van Wering ER, Janka-Schaub GE, et al: **Cellular drug resistance profiles in childhood acute myeloid leukemia: differences between FAB types and comparison with acute lymphoblastic leukemia.** *Blood* 2000, **96:**2879-2886.

122. Aigner J, Villatoro S, Rabionet R, Roquer J, Jimenez-Conde J, Marti E, Estivill X: **A common 56-kilobase deletion in a primate-specific segmental duplication creates a novel butyrophilin-like protein.** *BMC genetics* 2013, **14:**61.

123. Lupski JR, Stankiewicz P: **Genomic disorders: molecular mechanisms for rearrangements and conveyed phenotypes.** *PLoS Genet* 2005, **1:**e49.

124. Venables JP, Strain L, Routledge D, Bourn D, Powell HM, Warwicker P, Diaz-Torres ML, Sampson A, Mead P, Webb M, et al: **Atypical haemolytic uraemic syndrome associated with a hybrid complement gene.** *PLoS medicine* 2006, **3:**e431.

125. Ionita-Laza I, Rogers AJ, Lange C, Raby BA, Lee C: **Genetic association analysis of copy-number variation (CNV) in human disease pathogenesis.** *Genomics* 2009, **93:**22-26.

126. Zhao Y, Marotta M, Eichler EE, Eng C, Tanaka H: **Linkage disequilibrium between two high-frequency deletion polymorphisms: implications for association studies involving the glutathione-S transferase (GST) genes.** *PLoS Genet* 2009, **5:**e1000472.

127. Fanciulli M, Petretto E, Aitman TJ: **Gene copy number variation and common human disease.** *Clin Genet* 2010, **77:**201-213.

128. Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, Gray-Owen SD, Arsenault AL, Kaushic C: **Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation.** *PLoS pathogens* 2010, **6:**e1000852.

129. Canny GO, McCormick BA: **Bacteria in the intestine, helpful residents or enemies from within?** *Infection and immunity* 2008, **76:**3360-3373.

130. Eckmann L, Kagnoff MF: **Intestinal mucosal responses to microbial infection.** *Springer seminars in immunopathology* 2005, **27:**181-196.

131. Ngaimisi E, Habtewold A, Minzi O, Makonnen E, Mugusi S, Amogne W, Yimer G, Riedel KD, Janabi M, Aderaye G, et al: **Importance of ethnicity, CYP2B6 and ABCB1 genotype for efavirenz pharmacokinetics and treatment outcomes: a parallel-group prospective cohort study in two sub-Saharan Africa populations.** *PLoS One* 2013, **8:**e67946.

132. Kwiatkowski DP: **How malaria has affected the human genome and what human genetics can teach us about malaria.** *American journal of human genetics* 2005, **77:**171-192.

133. Bentley RW, Pearson J, Gearry RB, Barclay ML, McKinney C, Merriman TR, Roberts RL: **Association of higher DEFB4 genomic copy number with Crohn's disease.** *The American journal of gastroenterology* 2010, **105:**354-359.

134. Aitman TJ, Dong R, Vyse TJ, Norsworthy PJ, Johnson MD, Smith J, Mangion J, Roberton-Lowe C, Marshall AJ, Petretto E, et al: **Copy number polymorphism in Fcgr3 predisposes to glomerulonephritis in rats and humans.** *Nature* 2006, **439:**851-855.

135. Ptak SE, Voelpel K, Przeworski M: **Insights into recombination from patterns of linkage disequilibrium in humans.** *Genetics* 2004, **167:**387-397.

136. Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, Gibbs RA, Hurles ME, McVean GA: **A map of human genome variation from population-scale sequencing.** *Nature* 2010, **467:**1061-1073.

137. Stranger BE, Forrest MS, Clark AG, Minichiello MJ, Deutsch S, Lyle R, Hunt S, Kahl B, Antonarakis SE, Tavare S, et al: **Genome-wide associations of gene expression variation in humans.** *PLoS Genet* 2005, **1:**e78.

138. Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, Maksimovic-Ivanic D, Stivala F, Mazzarino MC, et al: **Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging.** *Aging* 2011, **3:**192-222.

139. Blenk S, Engelmann J, Weniger M, Schultz J, Dittrich M, Rosenwald A, Muller-Hermelink HK, Muller T, Dandekar T: **Germinal center B cell-like (GCB) and activated B cell-like (ABC) type of diffuse large B cell lymphoma (DLBCL): analysis of molecular predictors, signatures, cell cycle state and patient survival.** *Cancer informatics* 2007, **3:**399-420.

140. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, et al: **Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling.** *Nature* 2000, **403:**503-511.

141. Schmidt S, Rainer J, Riml S, Ploner C, Jesacher S, Achmuller C, Presul E, Skvortsov S, Crazzolara R, Fiegl M, et al: **Identification of glucocorticoid-response genes in children with acute lymphoblastic leukemia.** *Blood* 2006, **107:**2061-2069.

142. Bhadri VA, Cowley MJ, Kaplan W, Trahair TN, Lock RB: **Evaluation of the NOD/SCID xenograft model for glucocorticoid-regulated gene expression in childhood B-cell precursor acute lymphoblastic leukemia.** *BMC Genomics* 2011, **12:**565.

143. Kayibanda B, Rosenfeld C, Goutner A, Bornkamm GW: **A new lymphoid cell line, Reh 6, with characteristics of non-T and non-B cells, lacking the Epstein-Barr virus genome and derived from human acute lymphoblastic leukemia.** *Intervirology* 1978, **9:**316-320.

144. Beesley AH, Rampellini JL, Palmer ML, Heng JY, Samuels AL, Firth MJ, Ford J, Kees UR: **Influence of wild-type MLL on glucocorticoid sensitivity and response to DNA-damage in pediatric acute lymphoblastic leukemia.** *Molecular cancer* 2010, **9:**284.

145. Kang H, Wilson CS, Harvey RC, Chen IM, Murphy MH, Atlas SR, Bedrick EJ, Devidas M, Carroll AJ, Robinson BW, et al: **Gene expression profiles predictive of outcome and age in infant acute lymphoblastic leukemia: a Children's Oncology Group study.** *Blood* 2012, **119:**1872-1881.

146. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, Hovi L, LeBlanc T, Szczepanski T, Ferster A, et al: **A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial.** *Lancet* 2007, **370:**240-250.

147. Chuk MK, McIntyre E, Small D, Brown P: **Discordance of MLL-rearranged (MLL-R) infant acute lymphoblastic leukemia in monozygotic twins with spontaneous clearance of preleukemic clone in unaffected twin.** *Blood* 2009, **113:**6691-6694.

148. Spijkers-Hagelstein JA, Schneider P, Hulleman E, de Boer J, Williams O, Pieters R, Stam RW: **Elevated S100A8/S100A9 expression causes glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia.** *Leukemia* 2012, **26:**1255-1265.

149. Chinnadurai R, Grakoui A: **B7-H4 mediates inhibition of T cell responses by activated murine hepatic stellate cells.** *Hepatology* 2010, **52:**2177-2185.

150. Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, de Lorenzo P, Valsecchi MG, Pieters R: **Gene expression profiling-based dissection of MLL translocated and MLL germline acute lymphoblastic leukemia in infants.** *Blood* 2010, **115:**2835-2844.

# ABBREVIATIONS

**%:** percent

**μg:** microgram

**μl:** microlitre

**μm:** micromolar

**aa:** amino acid

**ALL:** acute lymphoblastic leukemia

**AML:** acute myeloid leukemia

**ATRA:** all-*trans* retinoic acid

**BCR:** B cell receptor

**BM:** bone marrow

**BTN:** butyrophilin

**BTNL:** butyrophilin-like

**CLL:** chronic lymphoblastic leukemia

**CML:** chronic myeloid leukemia

**CNV:** copy number variant

**DNA:** deoxyribonucleic acid

**EDTA:** ethylenediaminetetraacetic acid

**ELISA:** enzyme-linked immunosorbent assay

**FACS:** fluorescence-activated cell sorting

**FBS:** fetal bovine serum

**FITC:** fluorescein isothiocyanate

**GC:** glucocorticoid

**h:** hour

**HL:** Hodgkin's lymphoma

**HRP:** horseradish peroxidise

**Ig:** immunoglobulin

**M:** molar

**mg:** milligram

**ml:** millilitre

**MLL:** mixed lineage leukemia

**mM:** millimolar

**NHL:** non Hodgkin's lymphoma

**nm:** nanometre

**NK:** natural killer

**nM:** nanomolar

**PAGE:** polyacrylamide gel electrophoresis

**PB:** peripheral blood

**PBS:** phosphate buffered saline

**PCR:** polymerase chain reaction

**PPAR:** peroxisome proliferator activated receptor

**RA:** retinoic acid

**RARα:** retinoic acid receptor alpha

**siRNA:** small interfering RNA

**TAE:** tris(hydroxymethyl)aminomethane-acetate-ethylenediaminetetraacetic acid

**TBE:** tris(hydroxymethyl)aminomethane-borate-ethylenediaminetetraacetic acid

**TCR:** T cell receptor

**TEMED:** N,N,N',N'-tetramethyl-ethane-1,2-diamine

Tris(hydroxymethyl)aminomethane

**v/v:** volume per volume

**w/v:** weight per volume

# ANNEX

# List of publications

**Aigner J**, Villatoro S, Rabionet R, Roquer J, Jiménez-Conde J, Martí E and Estivill X. A common 56-kilobase deletion in a primate-specific segmental duplication creates a novel butyrophilin-like protein. *BMC genetics*. 2013; 14:61.

**Aigner J,** Nordlund J, Martí E and Estivill X (2013). "Butyrophilin-9 (*BTNL9*), a novel glucocorticoid sensitive gene promotes resistance in *MLL-AF4* rearranged acute lymphoblastic leukemia (ALL)". *Manuscript submitted.*

# Communications at meetings

**Oral Presentation at the IV CRG Student Symposium, Barcelona Nov. 2011**
**Title: FUNCTIONAL IMPACT OD A COMMON COPY NUMBER VARIANT IN GLUCOCORTICOID INDUCED APOPTOSIS**

**Poster presented at the 60th annual meeting of "The American Society of Human Genetics", Washington, Nov. 2010**

**FUNCTIONAL IMPACT OD A COMMON COPY NUMBER VARIANT IN GLUCOCORTICOID INDUCED APOPTOSIS**

J. Aigner, R. Rabionet, S. Villatoro, L. Armengol, E. Marti, X. Estivill

**Poster presented at the Wellcome Trust Meeting "Genomics of Common Diseases", Hinxton, UK, Sept. 2009**

**A COMMON COPY NUMBER VARIANT ON CHROMOSOME 5 GENERATING A CHIMAERIC GENE IS A NEW SUSCEPTIBILITY VARIANT FOR STROKE**

J. Aigner, R. Rabionet, S. Villatoro, L. Armengol, M. Garcia-Aragones, J. Roquer, E. Cuadrado, J. Montaner, I. Fernandez, A. Carracedo, E. Marti, X. Estivill

**Poster presented at the American Society of Human Genetics, Honolulu, US, Oct. 2009**

**A CNV IN CHROMOSOME 5 GENERATING A CHIMAERIC GENE IS A COMMON PROTECTIVE VARIANT FOR STROKE** R. Rabionet, J. Aigner, S. Villatoro, L. Armengol, M. García-Aragonés, E. Cuadrado-Godia, J. Jiménez-Conde, A. Ois, A. Rodríguez-Campello, J. Roquer, I. Fernández-Cadenas, J. Montaner, A. Carracedo, E. Martí, X. Estivil