The Role of NMDA-Receptors (NMDARs) and NMDAR Antagonists in Murine T- and B-Lymphocyte Function

Dissertation

Zur Erlangung des akademischen Grades

doctor rerum naturalium

(Dr. rer. nat.)

genehmigt durch die Fakultät für Naturwissenschaften

der Otto-von-Guericke-Universität Magdeburg

von M.Sc. Narasimhulu Simma

geb. am 16.08.1981 in Srikakulam, India

Gutacher: Prof. Dr. rer. nat. Ursula Bommhardt

Prof. Dr. Dr. Sven Meuth

eingereicht am: 23.06.2015

verteidigt am: 10.12.2015

Acknowledgements

It gives me immense pleasure to thank my mentor Prof. Dr. rer. nat. Ursula Bommhardt for her precious scientific suggestions and professional guidance that helped me a lot throughout the whole project. She was more than a modest supervisor of my PhD thesis; she really took care of my professional life and fostered my knowledge through scientific discussions. Finally, I would like to thank her for her never-ending motivation and encouragement throughout my research work.

I am also very grateful to Prof. Dr. med. Burkhart Schraven, not only for giving me the opportunity to work in the Institute, but also for his continuous and critical support which helped me to develop my scientific work in a fruitful way.

I would like to thank Dr. Martin Heine for enriching my scientific knowledge and valuable support throughout my research as collaboration partner.

I would like to thank my colleagues Tanima Bose, Theresa Lowinus, Judith Mankiewicz, Sascha Kahlfuss, and my colleague and friend A. Varma for their support and help in correcting the thesis. I also would like to thank Gabi Weitz for her technical support during my project and all IMKI colleagues, technicians and scientists for their helpful scientific discussions and advice.

I would like to thank my friends Aditya, Koteswara Rao, Sai Srinivas and Pavan for their continuous support during my Magdeburg life.

Finally, I would like to thank my family and my beloved parents for their ever-lasting support throughout of my life.

Table of Contents

Summary

N-methyl-D-aspartate receptors (NMDARs) are voltage- and ligand-gated ion channels and key glutamate receptors in the brain. NMDARs play an important role in neuronal development, plasticity and excitotoxicity and are implicated in several neurological disorders as in Parkinson's and Alzheimer's disease, depression or schizophrenia. The blockade of NMDAR activity by NMDAR antagonists MK801, memantine and ifenprodil has been used to elucidate neuronal NMDAR function. Memantine is currently applied in treatment of advanced Alzheimer's dementia. Interestingly, NMDARs were reported to be expressed on human T cells and murine thymocytes and their presumable inhibition by NMDAR antagonists was shown to influence several cellular responses. The major goals of this study were to investigate NMDAR expression in murine CD4+ T helper (Th) and B cells and how NMDAR antagonists would modify their function. The expression of NMDAR subunits at mRNA level was detected in thymocytes, resting and activated Th cells as well as B cells. However, several methodical approaches to detect functional NMDARs at protein level remained inconclusive. Analysis of NMDAR knockout mice showed that the reported and by us assumed expression of NMDARs in murine thymocytes and T cells is questionable as the used and described NMDAR antibodies turned out to be unsuitable for NMDAR detection in lymphocytes. Moreover, thymocytes from NMDAR knockout and wildtype mice were inhibited to the same degree by NMDAR antagonists, strongly suggesting a cross-reactivity of NMDAR antagonists with other ion channels. Within our collaborative project, these were identified to be $K_v1.3$ and $K_{Ca}3.1$ potassium channels. NMDAR antagonists significantly reduced Th cell proliferation in a drug concentration- and TCR stimulus-dependent manner. This correlated with a reduced activation of PLCγ1, Erk1/2, Akt-mTOR-S6 and NFATc1 and thus important TCR-induced signalling pathways. B cells showed comparable responses to NMDAR antagonists. BCR-induced and also LPS/TLR4-induced proliferation and activation of Erk1/2, Akt-S6 and NFATc1 were strongly attenuated in the drugs' presence. In addition, IgM and IgG secretion of LPS-activated B cells was abolished. However, the frequency of BCR/CD40-stimulated B cells producing the immunosuppressive cytokine IL-10 was significantly enhanced by ifenprodil. Hence, NMDAR antagonists may be of high therapeutic potential to suppress/modulate T- and B-cell responses in autoimmune diseases or pathological systemic inflammation. Furthermore, the additional (possibly beneficial) side effects of NMDAR antagonists, like memantine, on T and B cells should be considered when these drugs are used in treatments of neuronal diseases.

Zusammenfassung

N-Methyl-D-Aspartat-Rezeptoren (NMDARs) sind spannungs- und liganden-gesteuerte Ionenkanäle und gehören zu den wichtigsten Glutamatrezeptoren im Gehirn. NMDARs spielen eine entscheidende Rolle in der neuronalen Entwicklung und Ausbildung neuronaler Plastizität und sind maßgeblich an neuronaler Exzitotoxizität beteiligt. Eine Fehlfunktion der Rezeptoren ist mit verschiedenen neuronalen Erkrankungen assoziiert, u.a. Schlaganfall, Parkinson, Alzheimer, Depression und Schizophrenie. Die Blockade der NMDAR-Aktivität durch Antagonisten, wie MK801, Memantin und Ifenprodil, hat zum Verständnis der NMDAR-Funktionen wesentlich beigetragen. Memantin wird derzeit als zugelassenes Medikament klinisch eingesetzt, um die Symptomatik fortgeschrittener Alzheimer Demenz einzudämmen. Interessanterweise wurde die Expression von NMDARs auch für humane T-Zellen und murine Thymozyten beschrieben und ihre angenommene Hemmung durch NMDAR-Antagonisten beeinflusste die Proliferation, Differenzierung und Apoptose der Zellen. Ein Ziel der vorliegenden Forschungsarbeit war der Nachweis funktioneller NMDARs in murinen CD4+ T-Helfer Zellen (Th) sowie B-Zellen. Zudem sollten die möglichen Auswirkungen von NMDAR-Antagonisten auf die Funktionen muriner Lymphozyten untersucht werden. Die Expression von NMDAR-Untereinheiten auf RNA-Ebene wurde in Thymozyten, ruhenden und aktivierten Th-Zellen sowie B-Zellen mittels RT-PCR nachgewiesen. Allerdings konnte trotz verschiedener experimenteller Methodik die Expression von NMDARs auf Proteinebene nicht überzeugend bestätigt werden. Analysen von Thymoyzten aus NMDAR-defizienten Mäusen zeigten, dass die zuvor beschriebene und von uns angenommene Expression funktioneller NMDARs in murinen Thymozyten und peripheren T-Zellen zumindest sehr fraglich ist, da nachgewiesen wurde, dass mehrere, hier und zuvor in der Literatur eingesetzte, NMDAR-Antikörper unspezifisch agieren und für den Nachweis von NMDARs in Lymphozyten ungeeignet sind. Zudem wurde die Proliferation von Thymozyten aus NMDAR-defizienten und Wildtyp-Mäusen durch NMDAR-Antagonisten im gleichen Ausmaß gehemmt. Dies zeigt, dass NMDAR-Antagonisten vornehmlich über die Kreuzinhibition anderer Ionenkanäle agieren. In unserem Kooperationsprojekt wurden $K_v1.3$ und $K_{Ca}3.1$ Kalium-Kanäle als „targets“ von NMDAR-Antagonisten aufgezeigt. NMDAR-Antagonisten blockierten die Th-Zellaktivierung und Expansion in Abhängigkeit ihrer Konzentration und der Stärke des TCR-Stimulus, wobei CD28-Kostimulation diese inhibitorische Wirkung weitgehend aufhob. Auf mechanistischer

Ebene schwächten NMDAR-Antagonisten die Aktivierung von PCLγ1, Erk1/2, Akt-mTOR-S6 und des Transkriptionsfaktors NFATc1 und somit die Aktivierung zentraler TCR-induzierter Signalkaskaden. Vergleichbare Ergebnisse wurden für B-Zellen erzielt. Interessanterweise wurde sowohl die BCR- als auch LPS/TLR4-induzierte Proliferation der B-Zellen durch die Antagonisten stark gehemmt, was mit einer verminderten Aktivierung von Erk1/2, Akt-S6 und NFATc1 in den B-Zellen korrelierte. Zudem verringerten die Antagonisten die IgM- und IgG-Produktion LPS-stimulierter B-Zellen. Hingegen wurde die Differenzierung BCR/CD40-stimulierter B-Zellen, die das immunsuppressive Zytokin IL-10 produzieren, durch Ifenprodil signifikant erhöht. Insgesamt implizieren meine gewonnenen Daten, dass NMDARs in (murinen) Lymphozyten keine bzw. nur eine untergeordnete Rolle spielen. NMDAR-Antagonisten könnten durch Kreuzinhibition von $K_v1.3$ und $K_{Ca}3.1$ Kanälen ein profundes therapeutisches Potential auf pathologische adaptive und angeborene Immunantworten haben. Durch die Suppression bzw. Modulation der T- und B-Zellfunktionen könnten sie z.B. die Immunantwort bei Autoimmunerkrankungen und systemischen Entzündungsreaktionen günstig beeinflussen. Andererseits sollten die beschriebenen (eventuell positiven) Auswirkungen auf T- und B-Zellen im Nebenwirkungs-profil von NMDAR-Antagonisten, wie Memantin, beachtet werden, wenn sie als Medikament bei der Behandlung neurologischer Erkrankungen eingesetzt werden.

1. Introduction

1.1 The adaptive immune system

To protect from infectious agents, vertebrates have developed a complex biological system of self-defence mechanisms, commonly called the immune system. The immune system is divided into innate (inborn) and adaptive (acquired) immunity. Innate immunity is non-specific and has no immunologic memory. Adaptive immunity recognizes and memorizes pathogens through antigen-specific mechanisms and provides long-term protection to the host against re-infection by the same pathogens.

Adaptive immune responses are mediated by B and T lymphocytes, which provide humoral and cellular responses, respectively. B cells produce antibodies (Abs), which are released into the blood stream and other body fluids. The Abs bind to viruses, microorganisms or toxins and can neutralize them leading to their elimination. Cellular immune responses are mediated by CD4+ or CD8+ T cells. Cytotoxic CD8+ T cells eradicate virus-infected host cells and take part in the elimination of tumor cells. CD4+ T helper (Th) cells regulate immune responses by secreting cytokines and providing co-stimulatory signals for the activation of macrophages, CD8+ T cells, B cells, and other immune cells (Murphy, 2012).

My thesis dealt with the analysis of signalling events in murine T and B cells with regard to the role of NMDARs (N-methyl-D-aspartate receptors), ionotropic glutamate receptors that are well characterized in neuronal cells and had been reported to be also functional in human T cells. Thus, in the following chapters short overviews on the development and activation of T and B cells will be given followed by an overview on NMDARs, their antagonists and proposed role in lymphocytes.

1.2 Development and function of T cells

Lymphocytes are responsible for the incredible specificity of adaptive immune responses to eliminate foreign antigens and help in activating other immune cells. They originate from multi-potent haematopoietic stem cells in the bone marrow. Some of the lymphoid progenitor cells leave the bone marrow and migrate via the blood stream into the thymus. In the thymus, the early progenitor cells receive signals to mature via several concrete differentiation steps into functional T cells, which leave the thymus to seed peripheral lymphoid organs like spleen and lymph nodes.

The maturation stages of thymocytes are distinguishable by the expression of stage-specific surface molecules. The early thymocyte progenitors lack CD4 or CD8 surface markers and therefore are called double negative (DN) thymocytes. DN cells arise in the cortical region of the thymus and in the mouse system are sub-divided into four stages, based on the expression of CD25 and CD44 molecules (Figure 1a).

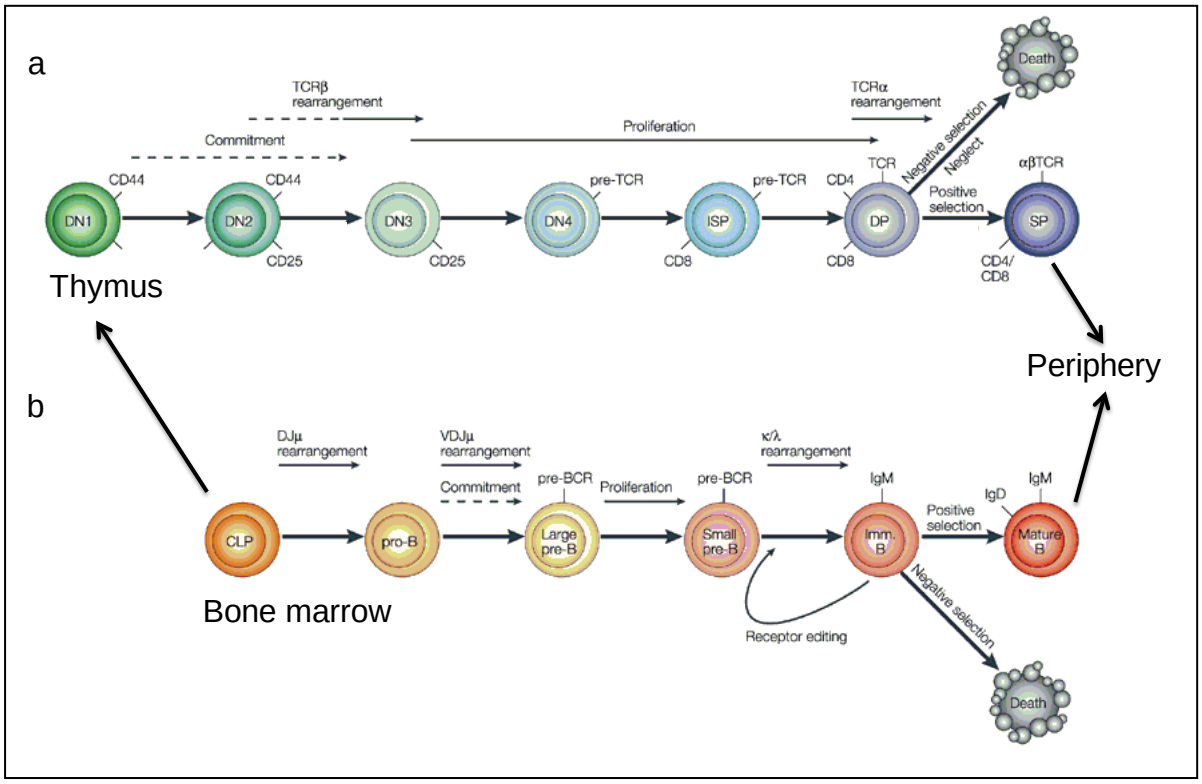


Figure 1. Schematic representation of T- and B-cell development. Common lymphoid progenitors (CLP) in the bone marrow give rise to both T- and B-cell lineages. **a**) T-cell development in the thymus. T-cell progenitors differentiate from double negative (DN) to double positive (DP) cells. At the DN2/DN3 stage, TCRβ-chain rearrangement takes place leading to the expression of a pre-TCR. Rearrangement of the TCRα-chain leads to the expression of mature α/β TCRs on DP thymocytes. DP cells are surveyed for the ligand binding affinity of their TCRs and undergo either positive or negative selection. DP cells that are positively selected will commit to either the CD4 or CD8 lineage. Negatively selected DPs die by apoptosis, thus avoiding the generation of autoreactive T cells. Mature single positive (SP) CD4+ and CD8+ T cells leave the thymus and migrate into secondary lymphoid organs. **b)** B cells develop from CLP in the bone marrow. Pro-B cells employ VDJμ rearrangement to generate a pre-BCR and differentiate into large pre-B cells. Signalling through the pre-BCR induces proliferation of large pre-B cells and their maturation into small pre-B cells. Rearrangement of κ- or λ-chains and their pairing with the IgM heavy chain generates a functional BCR on immature B cells. Are these positively selected, they migrate into the spleen to express IgD and finish maturation. Immature B cells that are not positively selected will rearrange further BCRs by receptor editing to clear positive selection or are negatively selected and die by apoptosis. Figure adapted from (Engel and Murre, 2001).

DN1 cells are CD44+CD25- and differentiate into CD44+CD25+ DN2 cells. Chemokines induce the migration of DN1/2 cells from the thymic cortex into the sub-cortex leading to the maturation of CD44-CD25+ DN3 cells. At the DN3 stage, cells start the rearrangement of the T cell receptor (TCR) β-chain, which combines with a surrogate pre-TCRα-chain (pTα) to form the pre-TCR, together with CD3γ, CD3δ, CD3ε and ζ chains of the CD3 complex. Failure to express a pre-TCR leads to the blockade of thymocyte development and DN3 cell cell death by apoptosis, a process known as β-selection (Germain, 2002; Swat et al., 1991). DN3 cells that cleared β-selection undergo proliferation via signals transduced by the pre-TCR, loose CD25 surface expression and mature into CD25-CD44- DN4 stage cells. Pre-TCR-induced survival and proliferation initiates arrest of further β-chain rearrangements (allelic exclusion) ensuring an individual β-chain on each cell. In DN4 cells, the expression of CD8 and CD4 surface markers is induced leading to the maturation of double positive (DP) thymocytes. At this point, extensive TCRα-chain rearrangements take place resulting in the formation of a heterodimeric 'mature' TCR bearing α- and β-chains (Figure 1a). DP cells undergo the second developmental checkpoint by testing the functionality of the TCR (αβ-selection) for MHC/self-peptide recognition and self-tolerance. The selection processes of DP cells are highly complex. In a simplified model, the strength and duration of TCR and co-receptor signals upon interaction of TCRs with MHCI- or MHCII-self-peptides presented on thymic epithelial cells and dendritic cells (DCs) determine whether DP cells die by so-called 'death of neglect' or by negative selection or whether they are positively selected and survive to further mature into single positive (SP) CD4+ Th or CD8+ cytotoxic T cells (lineage commitment). Importantly, these processes generate a self-tolerant and functional T-cell pool with an immense TCR repertoire that is able to protect the organism from a plethora of foreign antigens (Klein et al., 2014; Starr et al., 2003).

Upon encounter of their cognate antigen displayed on MHCI/II molecules of antigen presenting cells (APC), naive peripheral T cells get activated, undergo clonal expansion and differentiate into discrete T effector cell subsets. Th cell subsets are marked by their specific cytokine profiles or in case of cytotoxic CD8+ T cells by the production of granzymes and perforins. Th cells also interact with B cells to foster their activation and differentiation and migrate into inflammatory sites of infection to activate other cell types, thus helping in clearing the infection. Some of the antigen-experienced effector T cells differentiate into antigen-specific memory T cells (Chaplin, 2006; Murphy, 2012) .

Depending on the TCR stimulus, cytokine environment and the induction of specific 'master' transcription factors, activated Th cells differentiate into various Th cell subsets such as Th1, Th2, Th17, and regulatory T cells (Tregs), which show a specific cytokine secretion profile and specific effector functions. For instance, Th1 cells secrete IL-2 (interleukin 2) and IFN-γ (interferon γ) and activate the killing mechanisms of macrophages to destruct ingested intracellular pathogens. Th1 cells also help in clearing the infection by activating and recruiting cytotoxic CD8+ T cells and NK cells into the inflamed tissues. Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 cytokines and are involved in the regulation B-cell proliferation, Ig (immunoglobulin) class switching and their differentiation into Ab secreting plasma cells. Th17 cells secrete IL-17 cytokines and deliver inflammatory responses in anti-microbial and fungal defences. Tregs, induced in the periphery (pTregs) or matured in the thymus (tTregs) secrete IL-10 and TGF-β (transforming growth factor β) and act as important regulators of peripheral self-tolerance, chronic inflammation and autoimmune disorders (Zhu and Paul, 2008).

Cytotoxic CD8+ T cells (CTL) are involved in the killing of virus infected cells and eradication of intracellular pathogens. By production of several soluble factors such as perforin, granzyme, granulysin, IFN-γ, and TNF-α (tumor necrosis factor-α), they are also crucial for the elimination of tumor cells. TNF-α also co-operates with IFN-γ in the activation of macrophages.

1.2.1 T-cell signalling

Ligtion of the TCR by antigen presented by DCs, macrophages or B cells results in the activation of a TCR-induced complex signalling network including second messengers, kinases, phosphatases, and adaptor molecules. These regulate the activation of central signalling pathways, which lead to the activation of the transcription factors NFAT (nuclear factor of activated T cells), NFκB (nuclear factor kappa light chain enhancer of activated B cells) and AP1 (activator protein 1), which, among others, control IL-2 transcription and T-cell activation.

The positively charged TCRαβ heterodimer associates with the negatively charged CD3 complex, which transmits signal transduction. The CD3 chains harbour in their cytoplasmic tails immunoreceptor tyrosine-based activation motifs (ITAMs) which consist of two conserved YXXL or YXXI motifs separated from each other by 6-8 amino acids. In total, the

TCR/CD3 complex contains 10 ITAMs, formed from three ITAMs in each ζ-chain and one ITAM in each CD3ε-, γ- and δ-chain (Chakraborty and Weiss, 2014; Smith-Garvin et al., 2009). TCR engagement induces the activation of the major Src kinase Lck (lymphocyte-specific protein tyrosine kinase) which phosphorylates the ζ-chain ITAMs (Isakov et al., 1995) leading to the recruitment of ZAP-70 (zeta-chain-associated protein kinase of 70 kD) and its phosphorylation and activation by Lck (Acuto et al., 2008; Yan et al., 2013) (Figure 2). Active ZAP-70 phosphorylates the adaptor proteins LAT (linker for activation of T cells) and SLP-76 (SH2-domain containing leukocyte protein of 76 kD) recruited to pLAT (Paz et al., 2001).

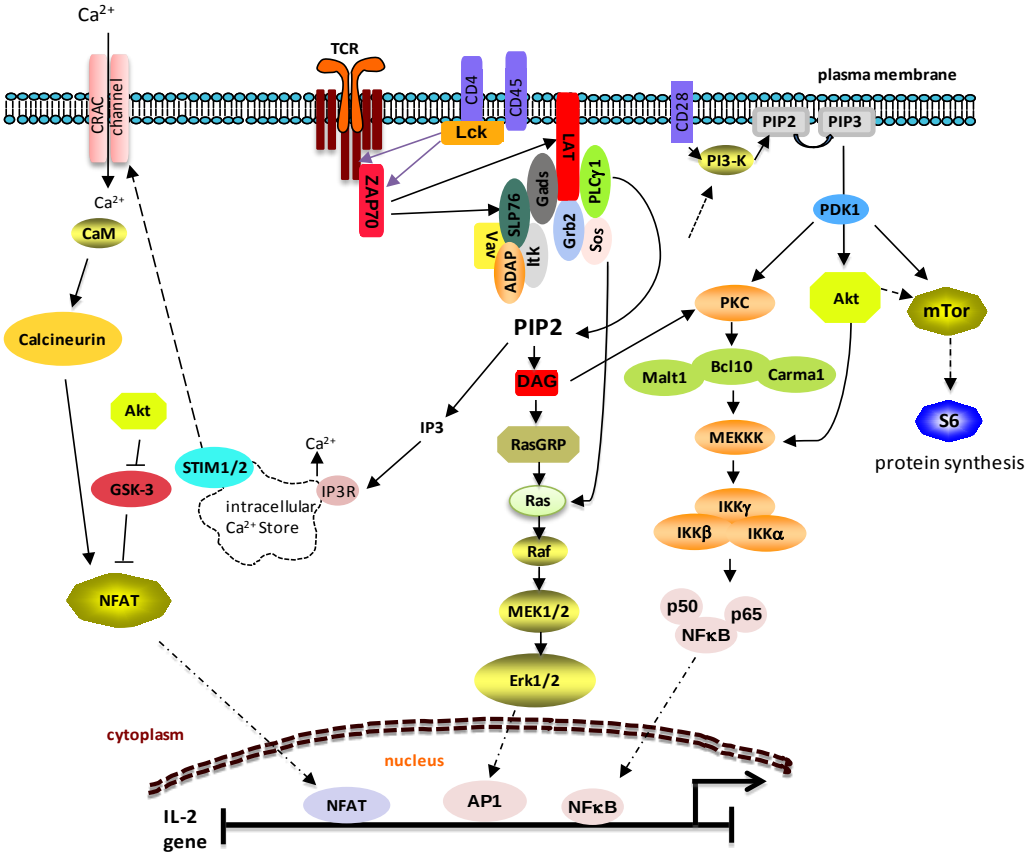


Figure 2. Schematic overview of TCR-induced signalling events. TCR-induced proximal signalling includes phosphorylation of the ITAMs of the TCR/CD3 complex by activated Lck, Zap70 activation by Lck and formation of a signalosome complex at phosphorylated LAT. More distal signalling events include formation of IP3 and DAG by activated PLCγ1, which lead to the activation of the Ca^{2+}/calcineurin/NFAT, Ras/Raf/Mek/Erk and PKC/NFκB cascades, respectively. TCR as well as CD28 ligation also induces activation of the lipid kinase PI3-K leading to the activation of Akt, mTOR and phosphorylation of ribosomal protein S6. Activated NFAT, AP1, and NFκB transcription factors initiate IL-2 cytokine transcription, which supports T-cell activation and proliferation. Figure adapted from (Love and Hayes, 2010; Nakayama and Yamashita, 2010).

Phosphorylated LAT recruits further signalling molecules to the plasma membrane including Gads (Grb2-related adapter downstream of Shc), PLCγ1 (phospholipase C gamma 1), Grb2 (growth factor receptor-bound protein 2) and Sos (son of sevenless), Itk (inducible T cell kinase), lipid kinase PI3-K (phosphoinositide 3-kinase), Vav (named after the sixth letter of the Hebrew alphabet) and the adaptor proteins Nck (non-catalytic region of tyrosine kinase adapter protein) and ADAP (adhesion and degranulation-promoting adapter protein) (Balagopalan et al., 2010; Fuller et al., 2011; Kliche et al., 2006; Liu et al., 1999; Sylvester et al., 2010). The assembled multi-protein LAT complex forms the Ca^{2+}-signalosome and signalling podium for activation of the MAPK (mitogen-activated protein kinase) Erk1/2 (extracelluar signal-regulated kinase 1 and 2) and PKC (protein kinase C). Activated PLCγ1 hydrolyzes the breakdown of the membrane lipid PIP2 (phosphatidylinositol 4, 5-biphosphate) into the second messengers IP3 (inositol 1, 4, 5-triphosphate) and DAG (diacylglycerol). IP3 activates the Ca^{2+}-dependent signalling pathway and DAG initiates activation of PKC and Erk1/2 (Smith-Garvin et al., 2009). These major signalling events induce the activation of NFAT, NFκB and AP-1, respectively (Figure 2).

1.2.2 The Ca^{2+} - calcineurin - NFAT pathway

In the cytoplasm, NFAT factors exist in the inactive phosphorylated form (Sharma et al., 2011). Their canonical activation requires elevated intracellular Ca^{2+}-levels and de-phosphorylation by the phosphatase calcineurin. IP3, generated by active PLCγ1, binds to IP3 receptors located in the endoplasmic reticulum (ER) membrane, leading to the release of Ca^{2+} from intracellular stores into the cytoplasm. Reduced Ca^{2+}-levels in the ER are sensed by ER high affinity calcium sensor proteins STIM1/2 (stromal interaction molecule 1/2) which mediate opening of the plasma membrane CRAC (calcium release activated channel) channels (Gwack et al., 2007; Hogan and Rao, 2015; Weiss, 2009). Influx of Ca^{2+} through CRAC channels induces the activation of Ca^{2+}/CaM (calmodulin)-dependent enzymes like calcineurin. Calcineurin activates NFAT factors through dephosphorylation of at least 13 phosphoserines in the regulatory region of NFAT, thereby promoting its translocation into the nucleus (Figure 2) (Robert et al., 2011). In my thesis, I focussed on the analysis of NFAT family member NFATc1 (NFAT2), which is transcriptionally induced and activated in naive Th cells upon TCR triggering (Chuvpilo et al., 2002). NFATc1 plays an important role in thymocyte development (Patra et al., 2013; Patra et al., 2006), the regulation of cytokine and

cytokine receptor expression, T-cell apoptosis, proliferation and Th cell differentiation (Rao et al., 1997; Rudolf et al., 2014; Serfling et al., 2007; Serfling et al., 2006).

1.2.3 The Ras-Erk-AP-1 pathway

The AP-1 transcription factor is a dimeric protein complex of Jun and Fos (FBJ murine osteosarcoma viral oncogene homolog) family transcription factors and its formation is dependent on Jnk (c-jun N terminal kinase) and Erk1/2 signalling. DAG, generated from PIP2 by active PLCγ1, activates PKC and the GTP exchange factor RasGRP (rat sarcoma guanyl-releasing protein) which is involved in the activation of Ras. Ras activates the MAPKinase cascade by binding to the serine/threonine kinase c-Raf (rapidly accelerated fibrosarcoma) through its Ras binding domain. Activated c-Raf dimerizes and activates MEK (mitogen activated protein/Erk kinase), the upstream kinase of Erk1/2. Erk1/2 regulates gene transcription either directly by phosphorylating and stabilizing the transcription factors cFos and cMyc or indirectly by phosphorylating Rsk (ribosomal S6 kinase) and Elk (ETS-like transcription factor). AP-1 in concert with NFAT functions as a transcriptional activator and initiates the expression of CD69, which is known as an early marker of T-cell transcriptional activation (Smith-Garvin et al., 2009).

1.2.4 Activation of the PI3-K-Akt-mTOR pathway

Besides triggering of the TCR (signal 1), T-cell activation requires additional signals provided by co-stimulatory receptors like CD28 (signal 2). CD28 binds to its ligands CD80 (B7-1) and CD86 (B7-2) expressed on the surface of activated APCs and induces signals that synergize with TCR-induced signalling events supporting T-cell activation, survival, and differentiation (Rudd et al., 2009). Both, TCR and CD28 ligation induce the activation of lipid kinase PI3-K, which converts PIP2 into PIP3. Membrane anchored PIP3 serves a docking site for PH (pleckstrin homology)-domain containing proteins like PDK1/2 (phosphoinositide-dependent protein kinase 1/2) and protein kinase B (PKB), also known as Akt (Figure 2). Akt is phosphorylated by PDK1 and mTORC2 (mammalian target of rapamycin complex 2) and is a central regulator of cell growth and survival. Akt signalling leads to the activation of mTOR and p70S6Kinase, which phosphorylates ribosomal protein S6, thereby fostering protein synthesis. Phosphorylation of GSK3β (glycogen synthetase kinase 3 β) by Akt inactivates GSK3β (Cross et al., 1995), which is one of the constitutively active NFAT kinases and a negative regulator of NFAT activation (Diehn et al., 2002). Once T cells are activated, their functional responses are further regulated through different cytokine signals (signal 3).

1.3 Development and function of B cells

B lymphocytes provide antigen-specific and long-term defence against different pathogens through the production of high affinity Abs of different isotypes. However, B cells can also induce or contribute to disease, for instance in autoimmune diseases by the production of autoantibodies as in MS (multiple sclerosis) or lupus erythematosus (Yanaba et al., 2008). Autoantibodies against neurotransmitter receptors or voltage-gated ion channels in the brain influence the opening behaviour of neuronal ligand- and voltage-gated ion channels (Kleopa, 2011) and lead to synaptic dysfunction. Such autoantibodies were observed in Rassmussen encephalitis (Rogers et al., 1994), Lambert Eaton myasthenic syndrome (Lennon et al., 1995) or anti-NMDAR encephalitis (Finke et al., 2013; Ramanathan et al., 2014; Sansing et al., 2007).

B cells differentiate from CLPs in the bone marrow. Pro-B cells form the earliest progenitors committed to the B-cell lineage (Figure 1b). They express two B-cell lineage specific transcription factors, E-protein E2A and EBF (early B-cell factor), which are required for formation of the pre-BCR (pre-B cell receptor) in pro-B cells (Melchers, 2005). With the help of RAG1/2 (recombinase activationg gene), E2A and EBF initiate gene rearrangements at the Ig heavy chain locus, usually in the μ region. Once a heavy μ chain is successfully formed, it is expressed on the surface with two surrogate chains called λ and VpreB and the two invariant chains Igα and Igβ forming the pre-BCR (ten Boekel et al., 1998). Expression of the heavy chain stops rearrangements in the heavy chain locus of the pro-B cell to prevent a B cell producing two receptors of different antigen specificities (allelic exclusion). Pro-B cells that fail to produce a functional heavy chain are eliminated by apoptosis. The pre-BCR complex provides signals for the rearrangement of the Ig light chain genes and allows a pro-B cell to proceed to the next stage of development. Once formed, pre-B cells initiate gene recombination in the Igκ or Igλ light chain locus. Successfully rearranged light chains are paired with the μ chains to form complete surface IgM molecules (van Zelm et al., 2007). At this stage, IgM in association with Igα and Igβ forms a functional BCR complex and B cells are called immature B cells (Figure 1b). The BCR is tested for autoreactivity and the signals generated from the BCR complex upon engagement with specific antigen dictate the fate of immature B cells. Cells with BCRs that strongly recognise autoantigens are eliminated by apoptosis, inactivated by immunological unresponsiveness (anergy) or immunological ignorance. In addition to these selection processes, autoreactive immature B cells can be

rescued by further gene rearrangements in their light chains to produce a new BCR that is not self-reactive (receptor editing). Immature B cells that are not strongly self-reactive will eventually transform into mature B cells and migrate into the spleen where they undergo final maturation (Figure 1b) (Murphy, 2012). Mature B cells are characterised by the expression of IgM and IgD molecules and receive survival signals from BCR engagement with antigen and BAFF (B cell activating factor) receptors (Kraus et al., 2004).

Activated naive B cells differentiate into several B-cell subsets that are involved in protective humoral immune responses. A subpopulation of B cells, called B10 cells, displays immunomodulatory functions by producing the immunosuppressive cytokine IL-10. B10 cells require for their formation BCR engagement and activation via the CD40 receptor or LPS (lipopolysaccharide) stimulation (Blair et al., 2009; Poe et al., 2011; Yanaba et al., 2009). They play a crucial role in preventing inflammatory and autoimmune pathologies and a lack of or inhibition of B10 cells has been associated with exacerbated experimental autoimmune encephalitis (EAE) (Fillatreau et al., 2002; Matsushita et al., 2008), collagen-induced arthritis (Mauri et al., 2003) or colitis in mice (Baba et al., 2015; Bouaziz et al., 2008; Candando et al., 2014).

1.3.1 B-cell signalling

The BCR complex comprises a transmembrane Ig and Igα and Igβ molecules for signal propagation (Burkhardt et al., 1991). BCR ligation leads to the phosphorylation of the ITAMs in the cytoplasmic tails of Igα and Igβ by the Src kinases Lyn (Lck/Yes novel tyrosine kinase) and Fyn (feline-yes related protein) (Kurosaki, 1999). The phosphorylated ITAMs recruit Syk (SH2 domain containing tyrosine kinase) and activated Syk phosphorylates the adaptor protein SLP-65 (SH2-domain containing leukocyte protein of 65 kD, also known as BLNK). SLP-65 forms a signalosome by recruiting several kinases and adaptor proteins such as Btk (Bruton's tyrosine kinase), PLCγ2 and Grb2 (Figure 3) (Ackermann et al., 2011; Rolli et al., 2002). PLCγ2 is activated by Btk and then hydrolyzes PIP2 into the second messengers IP3 and DAG. These lead to Ca^{2+}-mobilization, activation of PKC, Ras/Raf/Mek/Erk1/2, NFAT factors and NFκB, as described above for T cells (Wienands and Engels, 2001).

The BCR functionally associates with the B cell co-receptor CD19 that upon ligation recruits PI3-K, Vav and Lyn to the phosphorylated tyrosine residues in its cytoplasmic tail (Wang et al., 2002). PI3-K, induced by CD19 or the BCR, initiates Akt-mTOR-S6 signalling which is

important for B-cell development, activation, growth and survival (Okkenhaug et al., 2014; Pierau et al., 2012; Wienands and Engels, 2001).

CD40 is another important receptor on B cells. Its ligation by CD40L, expressed on activated Th cells, induces cell cycle and cell survival promoting genes; it also regulates Ig class switching and somatic hypermutation in B cells. CD40 signalling includes activation of Erk1/2, Jnk and p38 kinases that are involved in the activation of the transcription factors Elk-1 (ETS-like transcription factor), AP-1 and ATF1 (activating transcription factor 1). CD40 triggering also induces activation of PI3-K, NFκB, and Jak-Stat (janus activated kinase-signal transducer activator of transcription) proteins (Harnett, 2004). As mentioned before, BCR plus CD40 signals are critical to induce B10 cell generation.

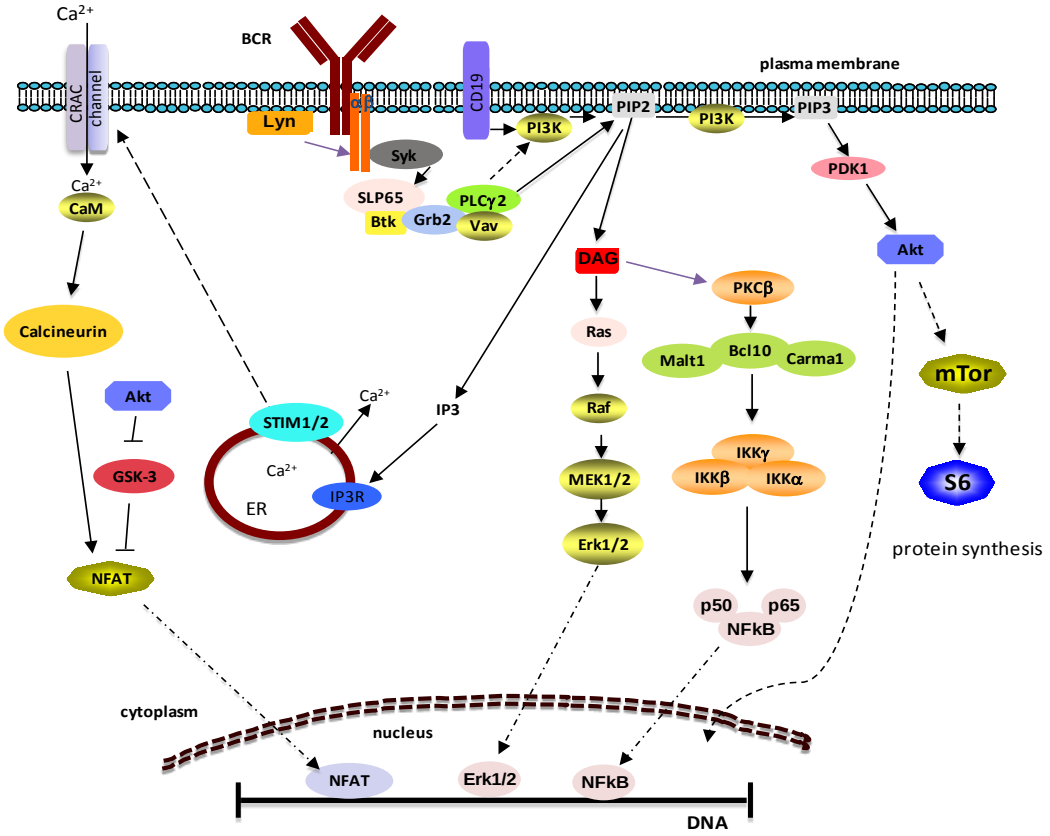


Figure 3. Schematic overview of B-cell signalling. The BCR complex consits of IgM and Igα/Igβ signal transducing molecules. Upon BCR ligation, the Src kinase Lyn is activated and phosphorylates the ITAMs of the BCR complex to initiate proximal signalling events. Syk recruited to pITAMs becomes activated and phosphorylates SLP65 (BLNK), which then recruits Btk, Grb2, Vav and PLCγ2. Activated PLCγ2 hydrolyzes PIP2 into DAG and IP3 second messengers. DAG is involved in the activation of PKC-NFκB and Ras-Raf-Mek-Erk1/2 cascades. IP3 mediates CRAC channel opening and NFAT activation. PI3-K is activated by BCR and CD19 ligation and initiates Akt-mTOR-S6 signalling. Activated transcription factors like NFAT and NFκB initiate changes in gene expression important for clonal proliferation and differentiation into plasma or memory B cells. Figure adapted from (Baba and Kurosaki, 2011).

1.4 TLR signalling

The innate immune response is the first line of defence to protect the host from invaded pathogens. Previously, innate immunity was considered as a system that only works through non-specific immune responses by phagocytic cells such as macrophages, neutrophils and DCs, which engulf and eliminate the microbial pathogen. Later, it was revealed that the fly Drosophila expresses special receptors for recognizing fungal infections, called Toll receptors (Akira et al., 2001). A human homolog of Toll was then shown to have the ability to induce the expression of inflammatory cytokines and co-stimulatory molecules (Janeway and Medzhitov, 2002). Until now, 13 members of Toll-like receptors (TLRs) have been identified in mice and 10 in humans, which are involved in the activation of innate immune cells and B cells. TLRs recognize PAMPs (pathogen associated molecular patterns) that are highly conserved motifs of pathogens. Every TLR has its individual pattern recognition specificity (Lee and Kim, 2007), for example TLR1, 2 and 6 recognize several bacterial components such as peptidoglycans, lipopeptides and lipoproteins, TLR3 binds double stranded RNA produced during viral infection (Alexopoulou et al., 2001) and TLR5 recognizes bacterial flagellin (Hayashi et al., 2001). TLR4 recognizes LPS of Gram-negative bacteria (Hoshino et al., 1999), which induces polyclonal activation of B cells.

TLRs recognizing PAMPs signal through adaptor molecules such as MyD88 (myeloid differentiation factor 88), TIRAP (TIR domain-containing adapter protein), TRIF (TIR domain-containing adapter inducing interferon-β), and TRAM (TRIF related adapter molecule). TLR3 and TLR4 activate MyD88- and TRIF-dependent pathways and induce type 1 interferon IFN-β. TLR-induced signalling results in the activation of IRAK1/4 (IL-1R associated kinase 1/4), TRAF6 (tumor necrosis factor associated receptor 6), Erk1/2, Akt and the transcription factors NF-κB, AP1, NFAT, and IRFs (interferon regulatory factors) (Kawai and Akira, 2006). TLR4 plays a major role in the initiation of inflammation and is a potent drug target to prevent severe sepsis, which is the primary cause of death amongst severely ill patients (Rauch et al., 2012).

In the part of my thesis dealing with the influence of NMDAR antagonists on T- and B-cell signalling, I used CD3, CD3 plus CD28 Abs and IgM $(Fab')_2$ and CD40 Abs or LPS for the polyclonal activation of T and B cells, respectively. Changes in the activation of Lck/Fyn, PLCγ1, Erk1/2, Akt-mTOR-S6, GSK3β, and NFATc1 were used as a read-out for the effects of NMDAR antagonists on the above described major signalling pathways in T and B cells.

These signalling molecules are crucial for B- and T-cell activation, and their inhibition by several pharmaceutical drugs, like rapamycin, cyclosporine A or Erk inhibitors, is in medical use or clinical trial to suppress pathological lymphocyte responses in various immune diseases.

1.5 Neuronal glutamate receptors

Glu (glutamate) is the major excitatory neurotransmitter in the CNS (central nervous system). GluRs (glutamate receptors) are involved in crucial neuronal functions such as neuronal development and synaptic plasticity, thereby providing the molecular basis for learning and memory. In pathophysiological conditions, they mediate excitotoxicity and neuronal degeneration. GluRs are classified into mGluRs (metabotropic) and iGluRs (ionotropic) receptors (Mayer, 2005).

mGluRs belong to the superfamily of G-protein-coupled receptors. They are composed of an extracellular domain binding Glu, a transmembrane region consisting of seven transmembrane domains and an intracellular region. The mGluR family encompasses eight receptor subtypes, which are classified into three groups (I, II and III) based on the amino acid sequence homology, agonist receptor pharmacology and intracellular signal transduction (Conn, 2003; Niswender and Conn, 2010). Group I receptors are mGlu1R and mGlu5R, Group II receptors comprise mGlu2R and mGlu3R, and Group III receptors contain mGlu4R, mGlu6R, mGlu7R, and mGlu8R. Glu binding to Group I receptors activates PLCγ1 leading to the activation of Ca^{2+}, Erk1/2, and PKC signalling. Group II and III receptors are negatively linked to adenyl cyclase and their activation reduces the levels of intracellular cAMP (adenosine 3-5 cyclic monophosphate) (Ferraguti and Shigemoto, 2006).

iGluRs are ligand-gated ion channels, which form homo- or heteromeric channels with four transmembrane domains. The main function of iGluRs is to mediate excitatory synaptic transmission in the CNS. They are classified into three groups based on their sensitivity to pharmacological agonists and structural properties: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid), KA (kainate) and NMDA (N-methyl-D-aspartate) receptors (Wisden and Seeburg, 1993). The AMPA and KA receptors induce synaptic transmission through permeability of Na^{2+} ions in response to Glu binding (Nakanishi, 1992). Within iGluRs, NMDA receptors (NMDARs) have distinctive features: a pre-requisite for the immediate binding of Glu and the co-agonist glycine/D-serine, a voltage-dependent Mg^{2+}-

block, and a high permeability for Ca^{2+}, which presents their major role in neuronal development, synaptic plasticity, learning, and memory.

1.6 NMDARs and their antagonists

NMDARs are heterotetrameric ion channels consisting of the obligatory GluN1 subunit and two homodimeric or heterodimeric subunits called GluN2A-D, GluN3 or GluN4 (Figure 4). When NMDARs bind Glu or aspartate and the co-agonists glycine or D-serine, they become activated and induce a depolarization of the cell membrane potential. Glu and NMDA bind to the GluN2 subunit and glycine/D-serine to the GluN1 subunit of NMDARs (Johnson and Ascher, 1987). Although glycine levels are more abundant than D-serine levels, several studies showed that endogenous D-serine is the major exogenous co-agonist for NMDAR-mediated neurotoxicity (Shleper et al., 2005).

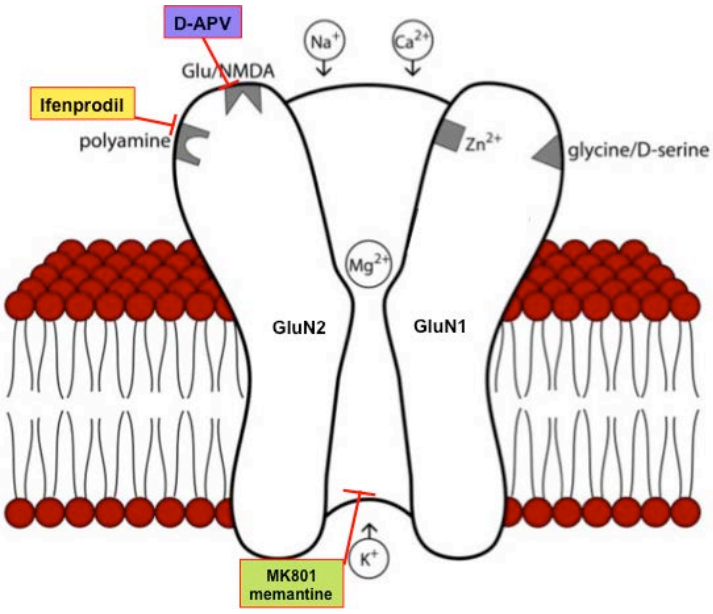


Figure 4. Structure and binding sites of NMDARs. The binding sites for Glu/NMDA, endogenous co-agonists glycine or D-serine, and antagonists are indicated. The competitive antagonist D-APV binds to the Glu/NMDA site and the uncompetitive antagonists MK801 and memantine in the open channel pore region. The non-competitive antagonist ifenprodil binds to the polyamine site of the GluN2 subunit. Figure modified from (Tomek et al., 2013).

In addition to binding of Glu and glycine/D-serine, activation of NMDARs requires an initial membrane depolarization by AMPA receptors to remove the Mg^{2+}-block of the channel pore, before the channel pore can be opened to permeate the influx of Ca^{2+} and Na^{+} and the efflux of K^{+} ions (Mayer, 2005; Mayer et al., 1984). The NMDAR opening kinetic depends on the subunit composition. Thus, NMDARs serve as co-incidence detectors and can initiate particular intracellular signalling events through the induction of intracellular Ca^{2+}-changes at small domains below the neuronal plasma membrane (Paoletti and Neyton, 2007). The localization and composition of NMDARs in the neuronal membrane are fundamental for the initiation of these signalling events (Groc et al., 2006).

In a pathophysiological role, NMDARs are associated with several neuronal disorders due to the induction of excitotoxic cell death as in epilepsy and stroke (Shohami and Biegon, 2014; Yuan et al., 2015), neurodegenerative diseases like Parkinson, Huntington (Daggett and Yang, 2013) and Alzheimer (Malinow, 2012), and psychiatric disorders like schizophrenia (Paoletti et al., 2013), and depression (Machado-Vieira et al., 2010). These diseases are connected to altered NMDAR activity and signalling after their activation. NMDAR-mediated Ca^{2+}-influx induces the activation of Ca^{2+}-regulated proteins such as CamKII (Ca^{2+}-Calmodulin regulated kinase II) and NFAT. NMDAR activity also results in the activation of PKC family members, Erk1/2 and PI3-K/Akt signalling and further transcription factors that orchestrate specific gene expression programs to regulate neuronal homeostasis, plasticity or cell death (Salter et al., 2009).

Since NMDARs are associated with several neuronal disorders, inhibition or modulation of NMDAR activity is of great clinical interest. *In vitro* blockade of NMDARs by several types of antagonists has been useful to understand their pharmacology and molecular function. NMDAR antagonists are classified into competitive, non-competitive and uncompetitive inhibitors. A competitive antagonist like D-APV competes with the agonist Glu or NMDA by binding to the same binding site of the GluN2 subunit of NMDARs (Figure 4). Non-competitive antagonists can act via binding to the active site or to an allosteric site of the receptor that is distinct from the active site or agonist binding site. Ifenprodil and ketamine are examples of non-competitive NMDAR antagonists. Ifenprodil binds to the GluN2B subunit (active site) of NMDARs, whereas ketamine binds to the allosteric site of the channel (Orser et al., 1997). Uncompetitive antagonists normally vary from competitive and non-competitive antagonists because they require a prior receptor activation and can bind to diverse allosteric binding sites. Memantine, which is clinically used to treat advanced Alzheimer's disease, and MK801, which is used in scientific research only, are prominent examples of uncompetitive antagonists and block opened NMDAR channels (Lipton, 2004).

Some NMDAR antagonists can cross-react with other neuronal ion channels. Memantine and MK801 were found to cross-react with α-7-nicotinic acetylcholine and serotonin receptors in heterologous expression systems and rat hippocampal neurons (Aracava et al., 2005; Iravani et al., 1999; Rammes et al., 2001). Memantine also cross-reacts with dopaminergic (D2) receptors in pituitary cells (Seeman et al., 2008). Ifenprodil blocks K^+ inward rectifier channels (Kobayashi et al., 2006) and ionotropic serotonin receptors (Barann et al., 1998).

1.7 GluRs in immune cells

In recent years it has become more obvious that there is a substantial cross-talk between the nervous and immune system. The neuronal chemical synapse and the immunological synapse show similarities in their structure and function and neuronal proteins have been detected in immune cells and *vice versa* (Boldyrev et al., 2005; Ganor and Levite, 2014*)*. As the function of these proteins in their 'odd' cell types is largely unknown, it is highly interesting to understand how for instance 'neuronal' proteins act in lymphocytes.

Besides the CNS, NMDAR expression has been detected in the heart, kidney, bone, and pancreas (Anderson et al., 2011; Bozic and Valdivielso, 2015; Chenu et al., 1998; Genever et al., 1999; Inagaki et al., 1995). The expression of NMDAR subunits (GluN1-GluN3B), AMPARs (GluR1-GluR3), KA receptors (KA1-KA2), and mGluRs (mGluR1-8) was found in 12 human cancer lines and human skin fibroblasts (Ganor and Levite, 2014). Interestingly, AMPARs (GluA3 subunit) (Ganor et al., 2003), group I mGluRs and NMDARs were also shown to be expressed in human PBLs (peripheral blood lymphocytes) and leukemic Jurkat T cells (Lombardi et al., 2001; Miglio et al., 2005). For murine cells, GluR mRNAs were detected in DN, DP and mature thymocyte subsets (Affaticati et al., 2011). Macrophages, neutrophils, DCs, and T cells were shown to release Glu (Affaticati et al., 2011; Pacheco et al., 2006) and Glu is found in lymphoid tissue and in blood (20-100 μM). Thus, it is attractive to conceive that GluRs have an important co-stimulatory role in lymphocyte function. Indeed, several groups reported a modulation of immunological responses through GluRs by applying different types of GluR antagonists (Ganor and Levite, 2014).

Glu-activated AMPARs in human T cells induced integrin-mediated adhesion to laminin and fibronectin (Ganor et al., 2003; Miglio et al., 2007). GluR3 was found to be expressed in T cells of multiple sclerosis (MS) patients and enhanced the proliferation of autoreactive T cells in response to myelin basic protein and myelin oligodendrocyte glycoprotein. In addition, GluR3 increased the chemotactic migration of T cells towards the chemokine CXCL12/SDF-1α (Sarchielli et al., 2007). In human lymphocytes, NMDARs were shown to be involved in the release of store-operated Ca^{2+} and the activation of Erk- and PKC-dependent signalling, which were inhibited in the presence of NMDAR inhibitor MK801 (Zainullina et al., 2011). In murine lymphocytes, NMDARs were found to increase intracellular Ca^{2+}-levels and the production of reactive oxygen species (ROS) (Boldyrev et al., 2004). The most comprehensive study on NMDARs in murine cells was performed by the Affaticati group.

They showed GluN1 localization in the immunological synapse formed between OT-II TCR transgenic (tg) thymocytes and DCs presenting cognate OVA (ovalbumin)-peptide. Addition of the NMDAR antagonists MK801 or memantine altered the duration of TCR-induced Ca^{2+}-flux and the apoptosis of DP thymocytes (Affaticati et al., 2011).

1.8 Aims of the study

The expression of functional NMDARs had been reported for human T cells, whereas the information on NMDARs in murine T cells was scarce when I started the thesis project. A potential function of NMDARs in B cells had not been addressed at all. Using Glu as a stimulating agent, in some publications it was not evident whether the described effects on T cells were due to mGluRs or iGluRs. Furthermore, the described influence of NMDAR antagonists on T-cell function was very exciting and opened the question how NMDAR activity would integrate into the T-cell signalling network.

Hence, the major aims of my studies were:

1) to prove the expression of NMDARs in murine T and B lymphocytes

2) to determine the influence of NMDARs on T- and B-cell activation and proliferation

3) to assess how NMDARs affect TCR-, BCR- and LPS/TLR4-induced signalling.

2. Material and Methods

2.1 Material

2.1.1 Cell culture material and reagents

RPMI 1640 medium with	
$NaHCO_3$ and stable glutamine and	Biochrom AG (Berlin, Germany)
10% FCS	Pan Biotech (Aidenbach, Germany)
5% glutamine solution	Biochrom AG
1% sodium pyruvate	Biochrom AG
100 U/ml penicillin/streptomycin	Biochrom AG
1% non-essential amino acids	Biochrom AG
50 µM β-mercaptoethanol	Gibco, Invitrogen (Darmstadt, Germany)

FCS was inactivated at $56^{O}C$ for 50 min, aliquoted and stored at $-20^{O}C$. Before addition to RPMI 1640 medium, the ingredients were sterile-filtered.

PBS without Ca^{2+} and Mg^{2+}	Biochrom AG
IL-4	ImmunoTools (Friesoythe)
LPS (E. coli 0111:B4)	Sigma-Aldrich (Taufkirchen)
phorbol 12-myristate 13-acetate (PMA)	Calbiochem (Darmstadt)
ionomycin	Calbiochem
brefeldin A	Calbiochem
monensin	Calbiochem

2.1.2 Antibodies (Abs)

2.1.2.1 Abs for cell stimulation

CD3ε (145-2C11)	BD Biosciences (Heidelberg)
CD28 (37.51)	BD Biosciences
IgM F(ab')$_2$ (α-IgM)	Jackson Immunoresearch (Hamburg)
CD40	Biolegend (San Diego, USA)

2.1.2.2 Abs for Western blot

Primary Abs:

phospho-PLCγ1^{Y783}	(rabbit polyclonal)	Cell Signalling (Frankfurt)
phospho-Src^{Y418}	(rabbit polyclonal)	Cell Signalling
phospho-mTOR^{S2448}	(rabbit polyclonal)	Cell Signalling
phospho-Akt^{S473}	(rabbit polyclonal)	Cell Signalling
phospho-Erk1/2$^{T202/Y204}$	(rabbit polyclonal)	Cell Signalling
phospho-GSK3β^{Ser9}	(rabbit polyclonal)	Cell Signalling
phospho-S6$^{S240/244}$	(rabbit polyclonal)	Cell Signalling
NFATc1 (7A6)	(mouse monoclonal)	Alexis Biochemicals (Lörrach, Germany)
actin	(mouse monoclonal)	Sigma-Aldrich
lamin B	(goat polyclonal)	Santa Cruz (Santa Cruz, USA)
NMDAR1/GluN1	(mouse polyclonal)	Synaptic systems (Göttingen)
		Alomone Labs (Jerusalem, Israel)

Secondary Abs:

peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L)	Jackson Immunoresearch
peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L)	Jackson Immunoresearch
peroxidase-conjugated AffiniPure rabbit anti-goat IgG (H+L)	Jackson Immunoresearch

2.1.2.3 Abs for flow cytometry

NMDAR1/GluN1	Synaptic Systems or Alomone Labs
IL-10-PE	eBiosciences (Frankfurt)
IFN-γ-FITC	eBiosciences
IgG2b-PE	eBiosciences
IgG2b-FITC	eBiosciences
anti-rabbit IgG (H+L)-PE (donkey polyclonal)	Jackson Immunoresearch
anti-mouse IgG (H+L)-FITC (goat polyclonal)	Jackson Immunoresearch

2.1.3 Radioactive material

^{3}[H]-Thymidine	MP Biomedicals (Heidelberg)

2.1.4 Inhibitors

ifenprodil	Tocris Biosciences (Bristol, UK)
memantine	Tocris Biosciences
MK801	Tocris Biosciences
D-APV	Tocris Biosciences

2.1.5 Chemicals and kits

37% acetic acid	Sigma-Aldrich
agarose	Applichem
40% acryl amide	Roth (Karlsruhe)
ammonium persulfate (APS)	Sigma (Munich)
bovine serum albumin (BSA)	Sigma
BCA protein assay kit	Pierce, Thermo scientific (Bonn)
chloroform	Roth (Karlsruhe)

complete protease inhibitor	Roche (Mannheim)
diethylpyrocarbonate (DEPC)	Sigma-Aldrich
dimethyl sulfoxoide (DMSO)	Sigma
dithiothreitol (DTT)	Sigma
ethylene glycol tetraacetic acid (EGTA)	Sigma
ethylene diamine tetraacetic acid (EDTA)	Sigma
enhanced chemiluminescence (ECL)	Thermo scientific (Karlsruhe)
ethanol	Roth
ethidium bromide	Roth
First Strand cDNA synthesis kit	Thermo scientific
glycine	Roth
Gene Ruler™ 100 bp Ladder	MBI Fermentas
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma
isopropanol	Roth
lauryl maltoside (LM)	Calbiochem
magnesium chloride ($MgCl_2$)	Roth
milk powder	Roth
methanol	Roth
NP-40	Sigma
phenylmethylsulfonylfluoride (PMSF)	Sigma-Aldrich
potassium chloride (KCl)	Sigma
Proteinase K	Sigma-Aldrich

protein standard marker (10-170 kDa)	Fermentas
roti-load 1 (4x)	Roth
sodium chloride (NaCl)	Roth
sodium azide (NaN3)	Sigma
sodium dodecylsulfate (SDS)	Roth
sodium orthovanadate (Na3VO4)	Sigma-Aldrich
sodium fluoride (NaF)	Sigma-Aldrich
sodium pyrophosphate (Na4O7P2)	Sigma-Aldrich
tetramethylethylenediamine (TEMED)	Sigma
TRIS	Roth
Trypan blue	Sigma
TRIzol	Life Technologies (Darmstadt)
Triton-X100	Roth
Tween 20	Roth

2.1.6 Miscellaneous material

1.5 ml reaction tubes	Eppendorf (Hamburg)
6-, 12-, 96-well plates	TPP (Trasadigen)
Elisa 96-well plates	Nunc Maxisorp, Thermo Fisher-Scientific (Marietta, USA)
15, 50 ml tubes	Greiner (Frickenhausen)
75, 175 cm^2 cell culture flasks	Corning Inc. (Wiesbaden)
Cuvettes 50-2000 µl (UVette 220-1600 nm)	Eppendorf (Hamburg)
cell scraper	Corning Inc.

cell strainer 100 μm Nylon BD FalconTM	BD Biosciences
5, 10, 25 ml pipettes	TPP
FACS tubes	BD Biosciences
FUJI MEDICAL X-RAY FILM-100 NIF	M. Hartenstein (Würzburg)
nitrocellulose (HybondTM-C Extra)	Amersham Biosciences
Whatman filter paper	Hartenstein
Pasteur pipettes	Roth
PCR tubes	Neolab (Heidelberg)
syringe filters (0.22 μm)	TPP

2.1.7 Instruments

autoMACS	Miltenyi Biotech (Bergisch Gladbach)
agarose gel electrophoresis chamber	Bio-Rad (Munich)
balance 440-47N	Kern & Sohn GmbH (Balingen-Frommern)
centrifuge 5415D (table centrifuge)	Eppendorf (Hamburg)
centrifuge Micro 200R (table centrifuge)	Hettich (Tuttlingen)
centrifuge Multifuge 3S-R	Heraeus (Hanau)
CO_2 incubator	Binder (Tuttlingen)
ELISA plate reader	Sunrise, Tecan, Männedorf, Switzerland
film developing machine	CAWO GmbH (Schrobenhausen)
flake ice machine	Thermo Electron Corporation (Erlangen)
gel documentation station Win32	Herolab (Wiesloch)
liquid scintillator 1450 Microbeta	Wallac Perkin Elmer (Turku)

magnetic stirrer	IKA (Staufen)
microwave	AEG (MICROMAT) (Halstenbenk)
Microscope Axioskop 2 plus	Zeiss (Jena)
PCR machine T3000 Thermocycler	Biometra (Göttingen)
PHD cell harvester	Inotech (Nabburg)
photo scanner perfection 4990	Epson (Meerbusch)
pH meter	Ino lab (Weilheim)
precision balance	OHAUS (Nänikon)
RC-5 super speed refrigerated centrifuge	Kendro Laboratory (Langenselb)
safety cabinet	Heraeus (Hera Safe HS 12) (Hanau)
shaker Duomax 1030	Heidolph (Kelheim)
spectrophotometer 3000	Biometra
thermomixer	Eppendorf
transilluminator	Messinstrumentebau (Erlangen)
vortex	IKA®-Labortechnik (Staufen)
Neubauer chamber	Roth
BD LSR Fortessa	BD Biosciences
water bath	GFL (Burgwegel)

2.1.8 Software

FlowJo v3.6.1	TreeStar (Ashland, USA)
Cell-Quest pro	BD Biosciences
1D 3.6 Image Quant	Kodak
Graphpad Prism 3.02	GraphPad Prism Inc. (CA, USA)

2.1.9 Mouse lines

C57BL/6 wildtype (wt)	bred at Animal Facilty, Medical Faculty, Magdeburg,
GluN1$^{flx/flx}$	provided bv Prof. R. Sprengel, Max-Planck-Institute for Medical Research, Heidelberg, Germany (Schwenk et al., 1995)
CMV-Cre deleter	provided bv Prof. R. Sprengel (Sprengel and Single, 1999)
IL-10 GFP knock-in-tiger	provided by Prof J. Hühn, HZI Braunschweig, Germany (Kamanaka et al., 2006)

All mice used in this study were on C57/BL6 background and 6-12 weeks or 16 weeks of age or were used as newborns within 8 hours after birth. Mice were bred at the Animal Facility of the Medical Faculty of the Otto-von-Guericke-University Magdeburg under pathogen-free conditions. All animal work was performed in compliance wih the German and local guidelines for the Use of Experimantal Animals.

2.2 Methods

2.2.1 Isolation of immune cells

2.2.1.1 Isolation of thymocytes, spleen and lymph node cells

Mice were sacrificed by CO_2 and thymus, spleen or lymph nodes were extracted and kept in supplemented RPMI medium at room temperature (RT). Organs were gently rubbed with a plastic syringe through a plastic cell strainer to obtain single cell suspensions in complete RPMI medium. An aliquot of cells was diluted with 0.1% Trypan blue in PBS to determine the cell concentration with a Neubauer cell-counting chamber.

2.2.1.2 Isolation of CD4+ T cells

Abs for cell isolation:

biotin anti-mouse CD8 (53-6.7)	BD Biosciences
biotin anti-mouse I-A/I-E (2G9)	BD Biosciences
biotin anti-mouse CD45R/B220 (RA3-6B2)	BD Biosciences
biotin anti-mouse TER-119/erythroid cells (Ly-76)	BD Biosciences
streptavidin micro beads	Miltenyi Biotech

Running buffer:

500 ml 1x PBS without Ca^{2+} and Mg^{2+}

10% BSA

2 mM EDTA

Isolation of CD4+ T cells was done by negative selection using AutoMacs technology (Miltenyi Biotech). Lymphocyte single cell suspensions were centrifuged at 1300 rpm (330 g Heraeus Multifuge 3S-R) for 5 min at $4^{O}C$. The supernatant was discarded and cells were suspended in running buffer (100 µl buffer for $1x10^7$ cells). A cocktail of depletion Abs was added to the cell suspension (0.2 µl of each Ab for $1x10^7$ cells) and cells were incubated for 20 min at $4^{O}C$ (gentle mixing with fingers was performed in between). Running buffer (2 ml for $1x10^7$ cells) was added for washing the cells. Samples were centrifuged at 1300 rpm for 5 min at $4^{O}C$ and the cell pellet was suspended in running buffer containing streptavidin beads (90 µl running buffer, 10 µl of streptavidin beads for $1x10^7$ cells). After gentle inversion of the tubes, cells were incubated for 15 min at $4^{O}C$. Running buffer (2 ml of buffer for 1 x 10^7 cells) was added and cells were centrifuged. The cell pellet was suspended in 1 ml running buffer for $1x10^8$ cells and to get rid of clumps, the cells were strained through a plastic cell strainer (100 µm) into a new Falcon tube. The isolation of CD4+ T cells was performed using an AutoMacs machine and selection of the 'Deplete' program. Cells were collected at the negative port of the Auto Macs. Isolated CD4+ T cells were spun down at 1450 rpm (450 g Heraeus Multifuge 3S-R) for 7 min at $4^{O}C$, the supernatant was discarded cells were suspended in supplemented RPMI medium and counted for further experimentation.

2.2.1.3 Isolation of B cells

B cell isolation kit from Miltenyi Biotech

Gey's solution:

Stock solution A:

35 g NH_4Cl

1.85 g KCl

1.5 g Na_2HPO_4, 2 H_2O

0.12 g KH_2PO_4

5 g glucose

add 1000 ml ddH_2O

Stock solution B:

0.42 g $MgCl_2$, 6 H_2O

0.14 g $MgSO_4$, 7H2O

0.34 g $CaCl_2$

add 100 ml ddH_2O

Stock solution C:

2.25 g $NaHCO_3$

add 100 ml ddH_2O

Final Gey's solution (100 ml): A+ B+ C+ ddH_2O = 20 ml+ 5 ml+ 5 ml+ 70 ml

Spleens of sacrificed mice were taken and kept in supplemented RPMI medium at RT. The spleen was gently rubbed through a plastic cell strainer (100 µm) with a plastic syringe to obtain a single cell suspension. Cells were centrifuged at 1300 rpm (330 g Heraeus Multifuge 3S-R) for 5 min at RT, the supernatant was discarded and cells were resuspended with 37^{O}C warm Gey's solution (3 ml for 1 spleen) for lysis of erythrocytes. Cells were again centrifuged for 5 min at 1300 rpm at RT. Cells were taken up in complete RPMI medium and the cell number was determined with 0.1% Trypan blue in PBS and a Neubauer counting chamber.

The isolation of B cells was done by negative selection using a B cell isolation kit and AutoMacs technology. Splenic cells were centrifuged at 1300 rpm for 5 min at 4^{O}C. The supernatant was discarded and cells were suspended in 40 µl running buffer/1x10^7 cells. From the provided Ab cocktail, 5 µl Ab cocktail/1x10^7 cells was added and cells were incubated for 15 min at 4^{O}C with gentle mixing in between. Thereafter, 30 µl of running buffer and 10 µl of streptavidin beads/1x10^7 cells were added and after gentle inversion of tubes, cells were incubated for 15 min at 4^{O}C. After addition of 2 ml of running buffer/1 x 10^7 cells and centrifuged at 1300 rpm for 5 min at 4^{O}C. The cell pellet was suspended in 1 ml running buffer/1x10^8 cells and cells were strained through a plastic cell strainer (100 µm) to remove clumps. The last two steps were repeated to recover all cells. The isolation of B cells was performed with the AutoMacs machine using the 'Deplete S' program. Negatively selected B cells were collected and spun down at 1450 rpm (450 g Heraeus Multifuge 3S-R) for 7 min at 4^{O}C. B cells were suspended in complete RPMI medium and the cell number was determined for further experimentation.

2.2.2 Isolation of RNA and RT-PCR

Freshly isolated or cultured CD4+ T cells or B cells were centrifuged at 1300 rpm (330 g Heraeus Multifuge 3S-R) for 5 min at RT. The supernatant was discarded and 1 ml of TRIzol reagent for 1x10^7 cells was added to the cell pellet. Cells were quickly lysed by pipetting up and down and the suspension was stored at -20^{O}C or RNA phase separation was performed. For this, the suspension was kept at RT for 5 min, 200 µl of chloroform/1 ml of TRIzol reagent was added, the tube was vigorously shaken by hand for a few seconds and then incubated for 3-10 min at RT. The sample was centrifuged at 13000 rpm for 15 min at 4^{O}C. The aqueous phase (upper layer) was collected without disturbing the lower phase and

transferred into a new Eppendorf tube. 500 µl of isopropanol/1x10^7 cells was added to the aqueous phase, the solution was mixed and incubated for 10 min at RT. Thereafter the solution was centrifuged at 13000 rpm for 15 min at 4^OC. The supernatant was discarded and the RNA pellet was washed with 1 ml 75% ethanol/1x10^7 cells by vortexing for a few seconds followed by centrifugation at 13000 rpm for 15 min at 4^OC. The supernatant was discarded and the above wash step was repeated. The RNA pellet was dried at RT for a few minutes and RNA was suspended in RNAse free DEPC H_2O. The RNA concentration was determined with a spectrophotometer. 1 µg of RNA was used for cDNA synthesis using the First-strand cDNA synthesis kit (Thermo Scientific) manufacturer's protocol. The cDNA was used for PCR analysis.

2.2.2.1 PCR primers and RT-PCR reaction

PCR primers for:

GluN1

Forward: 5`-TGTGTCCCTGTCCATACTCAAG-3´

Reverse: 5`-GTCGGGCTCTGCTCTACCACTC-3´

GluN2A

Forward: 5`-GGAGAAGG GTACTCCAGCGCTGAA-3´

Reverse: 5`-AGTCTGTGAGGAGATAAAAT CCAGC-3´

GluN2B

Forward: 5`-GCAAGCTTCTGTCATGCTCAACATC-3´

Reverse: 5`-GCTCTGCA GCTTCTTCAGCTGATTC-3´

GluN2D

Forward: 5`-CTTGGCTCCTCCACAGAGCAACAGC-3´

Reverse: 5`-CCTCTTCTGCCGCCCGGAAAACAGG-3´

IL-10

Forward: 5`-TGCCTTCAGTCAAGTGAAGACT-3´

Reverse: 5`-AAACTCATTCATGGCCTTGTA-3´

GAPDH

Forward: 5`-CAAGGTCATCCATGACAACTTTG-3´

Reverse: 5`-GTCCACCACCCTGTTGCTGTAG-3´

Actin

Forward: 5`-CCAGGTCATCACTATTGGCAAGGA-3´

Reverse: 5`-GAGCA GTAATCT CCTTCTGCATCC-3´

All PCR primers were purchased from APARA Bioscience (Jena, Germany), Taq polymerase and other PCR regents were obtained from Promega (Madison, USA).

RT-PCR reaction

GluN1 RT-PCR

PCR components	**concentration**	**quantity**
cDNA	0.5-1 µg	x µl
forward primer	10 µM	0.4 µl
reverse primer	10 µM	0.4 µl
dNTPs	10 mM	0.4 µl
$MgCl_2$	25 mM	2.0 µl
5 x Go green reaction buffer	5 x	4.0 µl
Taq DNA polymerase	5U/µl	0.1 µl
DEPC H_2O	-	12.7 µl

PCR program:

1. initial denaturation	$94^{O}C$	5 min	
2. denaturation	$94^{O}C$	1min	} 45 cycles
3. annealing	$61^{O}C$	1 min	
4. elongation	$72^{O}C$	1 min	
5. elongation	$72^{O}C$	5 min	
6. pause	$4^{O}C$	∞	

RT-PCR for NMDAR subunits, IL-10 and GAPDH:

PCR	annealing temp.	cycles	expected size of PCR fragment
GluN2A	54^{O}C	40	280 bp
GluN2B	67^{O}C	40	220 bp
GluN2D	57^{O}C	45	298 bp
IL-10	60^{O}C	40	346 bp
GAPDH	58^{O}C	30	496 bp

2.2.3 Isolation of DNA

DNA isolation buffer (tail lysis buffer)

50 mM Tris, pH 8.0
100 mM EDTA
100 mM NaCl
1% SDS

in ddH_2O

Isolated thymocytes were centrifuged at 1300 rpm for 5 min at 4^{O}C. Cells were lysed in 400 µl tail lysis buffer/1x10^{6} cells supplemented with 10 µl Proteinase K (20 mg/ml) for 10-20 min at 55^{O}C and shaking at 500 rpm. 400 µl isopropanol was added and after mixing the DNA was fished with a moulded glass Pasteur pipette. The Pasteur pipette tip with the attached DNA was dried at RT for a few minutes and sprayed with 70% ethanol for DNA washing. After air drying the Pasteur pipette tip was broken into 200 µl ddH_20 in Eppendorf tube. The DNA was dissolved from the broken glass tip by incubation at 60^{O}C for 20 min and 500 rpm rotation. The DNA concentration was determined by spectrophotometer and DNA was used for immediate PCR reactions or stored at -20^{O}C.

2.2.3.1 PCR of genomic DNA

Primers for:

GluN1^{flx}

P1-forward: 5`-CTGGGACTCAGCTGTGCTGG-3´

P2-reverse: 5`-AGGGGAGGCAACACTGT GGAC-3´

GluN1 deletion

P3-forward: 5`-GAGAAAGACATGGGGCATTATCC-3´

P2-reverse: 5`-AGGGGAGGCAACACTGT GGAC-3´

CMV-Cre

Forward: 5`-ACGACCAAGTGACAGCAATG-3´

Reverse: 5`-CTCGACCAGTTTAGTTACCC-3´

Actin

Forward: 5`-CCAGGTCATCACTATTGGCAAGGA-3´

Reverse: 5`-GAGCAGTAATCTCCT TCTGCATCC-3´

PCR for GluN1^{flx} and GluN1^{wt} alleles

PCR components	**concentration**	**quantity**
genomic DNA	0.5-1 µg	x µl
P1-forward	10 µM	1.0 µl
P2-reverse	10 µM	1.0 µl
dNTPs	10 mM	0.4 µl
$MgCl_2$	25 mM	2.0 µl
5x Go green reaction buffer	5x	4.0 µl
Taq DNA polymerase	5U/µl	0.1 µl
DEPC ddH_2O	-	9.5 µl

PCR programme:

1. initial denaturation	95^{O}C	5 min	
2. denaturation	95^{O}C	1 min	} 35 cycles
3. annealing	58^{O}C	30 sec	
4. elongation	72^{O}C	50 sec	
5. elongation	72^{O}C	5 min	
6. pause	4^{O}C	∞	

PCR for GluN1 deletion

PCR	annealing temp.	cycles	expected size of PCR fragment
GluN1 deletion (deletion of GluN1 exons 11-18; P3, P2 primers)	58^{O}C	35	780 bp
CMV-Cre tg	60^{O}C	34	350 bp
actin	58^{O}C	30	240 bp

2.2.4 Agarose gel electrophoresis

50 x TAP

242 g	Tris
57.1 ml	37% acetic acid
37.2 g	EDTA (titration complex 3)

add 1000 ml ddH_2O, pH 8.0

1% agarose gel

100 ml	1x TAP
1 g	agarose
10 mg/ml	ethidium bromide
Gene Ruler™	100 bp ladder

1% agarose was added to 1 x TAP buffer, the mixture was boiled in a microwave and 10 µl ethidium bromide was added before casting the agarose into a gel chamber. 10 µl of the PCR samples were loaded onto the gel agarose, which was run at a constant voltage of 100 V for 25-30 min. PCR DNA fragments were visualized under an UV gel documentation system (Herolab) and the size of PCR products was identified by the Gene ruler 100 bp ladder.

2.2.5 Proliferation assay

2.2.5.1 T-cell proliferation

Thymocytes or CD4+ T cells ($0.5\text{-}1x10^5$) were stimulated with plate-bound CD3 Abs (3 or 10 µg/ml) or CD3+CD28 Abs (3 and 5 µg/ml) in the presence or absence of NMDAR inhibitors in complete RPMI 1640 medium/10% FCS in triplicates in 96-well plates. ^{3}[H]-Thymidine (0.2 µCi/well) was added after 24 h of culture for 16 h. Cells were harvested using a PHD cell harvester (Inotech) and incorporated ^{3}[H]-Thymidine was determined with liquid scintillation counting using a liquid scintillator 1450 Microbeta Wallac machine (Perkin Elmer).

2.2.5.2 B-cell proliferation

Splenic B cells ($0.5\text{-}1x10^5$) were stimulated with soluble α-IgM or LPS (10 µg/ml). Cells were cultured in the presence or absence of NMDAR inhibitors in complete RPMI 1640 medium/10% FCS in triplicates in 96-well plates. ^{3}[H]-Thymidine (0.2 µCi/well) was added after 24 h. ^{3}[H]-Thymidine incorporation was determined as described for T cells.

2.2.6 Western blot analysis

10 x electrophoresis buffer

0.25 M	Tris base
1.92 M	glycine
1%	SDS (w/v)

in ddH_2O

10 x blotting buffer

1.92 M	glycine
0.25 M	Tris Base
20 %	methanol

in ddH_2O

10 x TBS

25 mM	Tris base
137 mM	NaCl

in ddH_2O

1 x TBST or wash buffer

10%	10 x TBS
0.05%	Tween-20

in ddH_2O

blocking buffer

1 x	TBST
5%	milk powder (w/v)

4 x upper gel buffer (UGB)

0.5 M	Tris
0.4%	SDS (w/v)

in ddH_2O, pH 6.8

4x lower gel buffer (LGB)

1.5 M	Tris
0.4%	SDS (w/v)

in ddH_2O, pH 8.8

SDS 8% polyacrylamide gel electrophoresis (PAGE) gel

11 ml	ddH_20
5 ml	40% acryl amide
5 ml	4 x LGB buffer
66 µl	10% APS
20 µl	TEMED

SDS 10% PAGE gel

10 ml	ddH_20
5 ml	40% acryl amide
5 ml	4 x LGB buffer
66 µl	10% APS
20 µl	TEMED

SDS PAGE stacking gel

5.5 ml	ddH_20
1.3 ml	40% acryl amide
5.0 ml	4 x UGB buffer
33 µl	10% APS
20 µl	TEMED

2.2.6.1 Preparation of total protein cell extracts

NP-40 lysis buffer

1%	NP-40
100 mM	NaCl
50 mM	HEPES pH 7.4
5 mM	EDTA
50 mM	NaF

Added freshly before use:

10 mM	sodium pyrophosphate
1%	lauryl maltoside (LM)
1 mM	phenylmethylsulfonylfluoride (PMSF)
1 mM	sodium orthovanadate
1 x	complete protease inhibitor

Protein extracts were prepared from thymocytes or lymph node CD4+ T cells, B cells or total brain. Single cell suspensions were washed once with 1 ml ice-cold PBS and centrifuged for 5 min at 1300 rpm. The cell pellet was lysed with ice-cold lysis buffer (100 µl for $1x10^7$ cells) and lysates were vortexed and incubated on ice for 30 min. Thereafter, lysates were centrifuged at 14000 rpm for 20 min at 4^OC. Supernatants were transferred into 1.5 ml Eppendorf tubes and used as total protein cell lysates. The protein concentrations were

determined by BCA protein assay kit. 1/4 volume of 4 x Roti-Load was added to the lysates which were boiled at 95^{O}C for 5 min and shaking at 500 rpm. Protein samples were stored at -20^{O}C. LM in the cell lysis buffer led to the disruption of membrane lipid rafts.

2.2.6.2 Preparation of cytoplasmic and nuclear protein extracts

Cytoplasmic protein extract (CE) buffer:

10 mM	KCl
10 mM	HEPES, pH 7.9
0.1 mM	EGTA, pH 7.9
0.1 mM	EDTA, pH 7.9

Added freshly before use:

1 mM	DTT
1 mM	sodium orthovanadate
1 x	complete protease inhibitor

Nuclear protein extract (NE) buffer:

420 mM	NaCl
20 mM	HEPES, pH 7.9
0.1 mM	EGTA, pH 7.9
0.1 mM	EDTA, pH 7.9

Added freshly before use:

1 mM	DTT
1 mM	sodium orthovanadate
1 x	complete protease inhibitor

CD4+ T cells or B cells (5x10^{6}) were harvested and washed once with 1 ml ice-cold PBS and centrifugation at 7500 rpm for 1 min at 4^{O}C. Cells were lysed with ice-cold CE buffer (70 µl for 1x10^{7} cells) and incubated for 2 min on ice. The solution was supplemented with 0.5% NP40 and incubated for 2 min on ice and was gently vortexed for a few seconds to get a transparent clear solution. The sample was centrifuged for 3 min at 14000 rpm and 4^{O}C, the collected supernatant was transferred into a new 1.5 ml Eppendorf tube and used as cytoplasmic protein extract (CE). The transparent pellet containing the nuclei was washed with ice-cold CE buffer (800 µl for 1x10^{7} cells) without disturbing the pellet and again centrifuged for 3 min at 14000 rpm and 4^{O}C. The supernatant was discarded and the wash

step was repeated. Afterwards ice-cold NE buffer (35 µl for $1x10^7$ cells) was added to the pellet, the Eppendorf tube was vortexed for a few seconds and kept on a rotating wheel at 4^OC for over night. The next day the sample was centrifuged for 20 min at 14000 rpm and 4^OC. The supernatant containing nuclear proteins was transferred into a 1.5 ml Eppendorf tube and used as nuclear protein extract (NE). Protein concentrations in CE and NE were determined with BCA protein assay kit. 1/4 volume of 4 x Roti-Load was added to the prepared lysates which were boiled at 95^OC for 5 min and shaking at 500 rpm. CE and NE were stored at -20^OC until use in Western blot.

2.2.6.3 SDS PAGE and Western blot

Protein lysates obtained from CD4+ T cells (5-15 µg), thymocytes (20 µg), B cells (15 µg) or brain (5 µg) were subjected to 8-10% SDS-PAGE. Separated proteins were transferred onto a nitrocellulose membrane, which was blocked with 5% non-fat milk powder in TBST for 1 h at RT and then washed three times for 5 min with wash buffer. The expression/activation of signalling proteins was analysed by incubating the membrane with primary Abs in TBST/5% BSA for 1 h or over night at 4^OC on a shaker. Thereafter, the membrane was washed three times for 5 min with wash buffer and incubated with HRP-coupled mouse anti-rabbit, goat anti-mouse or donkey anti-goat secondary Abs in TBST/5% milk for 1 h at RT. After three washes for 10 min with wash buffer, ECL was added and the membrane was exposed to X-ray film. For quantification, immune reactive bands on the film were scanned with a photo scanner (Epson perfection 4990) and analysed with 1D 3. 6 Image Quant software (Kodak).

2.2.7 Flow cytometry

FACS buffer: 0.5% BSA in 1 x PBS without Ca^{2+} and Mg^{2+}

FoxP3 staining buffer kit (BD Biosciences)

To measure intracellular protein expression of NMDAR subunits, ~2 $x10^6$ thymocytes or lymphnode cells were first stained for surface expression of CD4 and CD8 for 20-25 min at RT in FACS buffer. Stained cells were washed with 1 ml PBS and centrifuged at 1300 rpm for 5 min at RT. Cells were resuspended in 400 µl fixation and permeabilization buffer provided in the FoxP3 staining buffer kit and incubated for 25-30 min at RT. Thereafter 500 µl permeabilization buffer was added to the fixed cells and cells were centrifuged. The cell pellet was taken up in 100 µl permeabilization buffer containing primary Abs directed against NMDAR subunits. After incubation for 30 min at RT, 500 µl permeabilization buffer was

added and cells were centrifuged. Resuspended cells were stained with secondary fluorochrome-coupled anti-rabbit Abs for 30 min at RT. 500 µl permeabilization buffer was added, cells were centrifuged and washed once with FACS buffer. Cells were measured with a LSR Fortessa and flow cytometric analysis was performed with Cell-Quest pro and FlowJo 7.6.5 software.

2.2.8 Intracellular cytokine staining and IL-10-GFP expression assay

Splenic B cells were stimulated with α-IgM+CD40 (10 +5 µg/ml), CD40 Abs alone (5 µg/ml), or LPS (10 µg/ml) for 48 h or 72 h. Ifenprodil (10 µM) was added once and at day 1. Before harvest, the cells were treated with IO (800 ng/ml) and PMA (500 ng/ml) for 4 h in the presence of Brefeldin A (3 µg/ml). Thereafter, cells were fixed and stained with IL-10-PE and IFN-γ-FITC Abs and isotype controls IgG2b-PE/FITC by using the FoxP3 staining buffer kit (eBiosciences) and manufacturer's protocol. The percentage of live B cells producing IL-10 or IFN-γ was determined with flow cytometry. B cells isolated from IL-10-GFP tiger mice (Kamanaka et al., 2006) were activated with α-IgM/CD40 Abs (10+5 µg/ml) or LPS (10 µg/ml) and cultured in 96-well plates. Ifenprodil (10 and 20 µM) was added at 21-25 h and cells were harvested either at day 2 or day 4. Before harvest, cells were re-stimulated with PMA (100 ng/ml) and IO (800 ng/ml) for 4 h in the presence of monensin (10 µg/ml). IL-10 or IFN-γ production and IL-10-GFP expression in FSC/SSC-gated live B cells was determined with flow cytometry.

2.2.9 ELISA

B cells were activated with LPS (10 µg/ml) or LPS plus IL-4 (20 ng/ml) in complete RPMI/10%FCS medium in triplicates in 96-well plates. Ifenpodil (10 µM) was added at day 1, day 2 or at day 3 and culture supernatants were taken at day 4. ELISA plates were coated over night with 50 µl goat anti-mouse Ig (Southern Biotech, Birmingham, AL, USA) at 1:500 dilution in 50 mM sodium carbonate buffer. After washing with PBS/0.05% Tween 20, the wells were blocked with PBS/5% BSA for 1 h. The supernatant samples were diluted in PBS/5% BSA and incubated in the coated wells for 2 h at RT. After washing, POD-coupled anti-mouse IgM or IgG (Sigma-Aldrich) was added at 1:250 dilutions in PBS/5% BSA for 1 h at RT, followed by substrate development with TMB reagent (BD Biosciences). The OD at 450 nm was determined with an ELISA reader (Sunrise, Tecan, Männedorf, Switzerland).

2.2.10 Statistical analysis

The data in graphs are given as mean values + standard deviation (SD), if not stated otherwise. Unpaired Student's *t* test and other statistics were performed in GraphPad Prism 3.02. Statistical significance was set as *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

3. Results

3.1. The function of NMDARs in murine T cells

3.1.1 Analysis of NMDAR expression in thymocytes and CD4+ T cells

Previous studies reported the expression of NMDARs subunits in thymocytes and human lymphocytes based on RT-PCR and flow cytometry analyses. GluN1 subunit localization in the contact zone of murine thymocyte-DC pairs was shown by confocal microscopy (Affaticati et al., 2011; Ganor et al., 2003; Lombardi et al., 2001). Thus, I initially performed RT-PCR to elucidate mRNA expression of the NMDAR subunits GluN1, GluN2A and GluN2B in murine lymphocytes. mRNA was isolated from total thymocytes, naive as well as CD3+CD28 Ab-activated peripheral CD4+ T cells and brain, for comparison (Figure 5). GluN1, GluN2A and GluN2B mRNAs were found in thymocytes and naive mature CD4+ T cells (Figure 5, left panel) and GluN1 mRNA expression was upregulated in CD4+ T cells after their stimulation with CD3+CD28 Abs for 24 and 48 h (Figure 5, right panel). In line with the publication of Affaticati et al., the RT-PCR results suggested that NMDARs are expressed in thymocytes, but also in peripheral naive and activated CD4+ T cells.

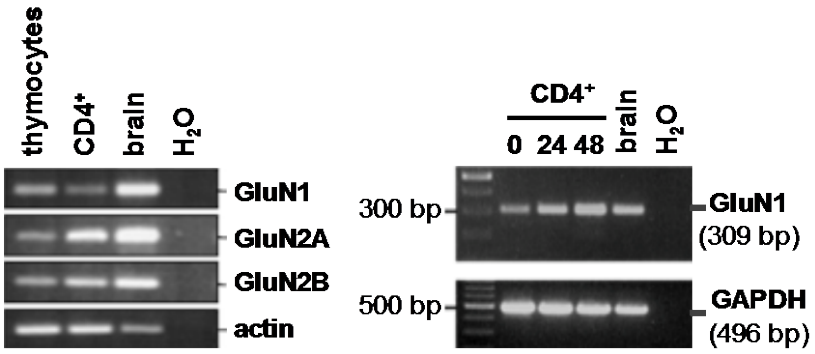


Figure 5. RT-PCR analysis of NMDAR subunit mRNA expression. mRNA expression of NMDAR subunits GluN1, GluN2A and GluN2B was analysed by RT-PCR in thymocytes, peripheral CD4+ T cells and brain (left panel), and GluN1 mRNA expression in CD3+CD28 Ab (3 and 5 µg/ml)-activated CD4+ T cells at the indicated time points in comparison to brain (right panel). Actin or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA expression served as RT-PCR control. Data are the representative of 2-3 experiments (Kahlfuss et al., 2014).

Subsequently, experiments were performed to detect protein expression of NMDAR subunits in CD4+ T cells. Isolated naive CD4+ T cells were intracellularly stained with GluN1, GluN2A and GluN2B Abs (from Alomone Labs) and analysed by flow cytometry (Figure 6). The obtained positive stainings for NMDAR subunits indicated protein expression of functional NMDARs in CD4+ T cells.

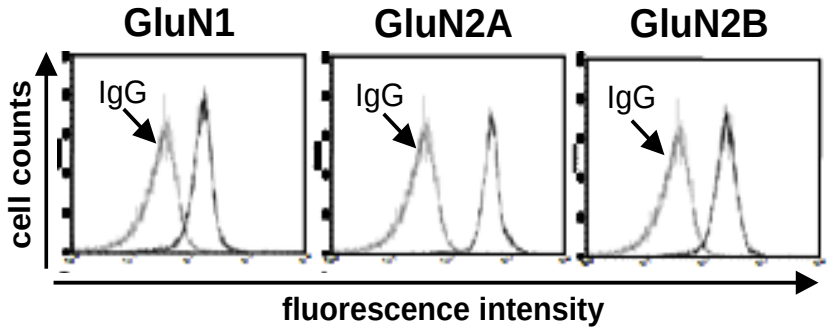


Figure 6. Expression of NMDAR subunits in CD4+ T cells at protein level. CD4+ T cells were intracellulary stained for GluN1, GluN2A and GluN2B (Abs from Alomone Labs) and analysed by flow cytometry. Histograms show NMDAR subunit (black line) and IgG2b isotype (grey line) staining and are representative for 3 independent experiments.

3.1.2. NMDAR antagonists inhibit T-cell proliferation

Since NMDARs seemed to be expressed in thymocytes and CD4+ T cells, we asked whether NMDARs play a role in CD4+ T-cell activation. To address this question, different NMDAR antagonists were used to inhibit NMDAR activity during T-cell stimulation. Isolated CD4+ T cells were stimulated with high (10 µg/ml) and low (3 µg/ml) concentrations of CD3 Ab in the presence or absence of the GluN2B antagonist ifenprodil and the open channel blockers MK801 and memantine. As shown in Figure 7, all three inhibitors significantly reduced T-cell proliferation in a concentration-dependent manner. Under strong stimulatory conditions with 10 µg/ml CD3 Ab (Figure 7a), T-cell proliferation was less inhibited by 3-10 µM ifenprodil compared to lower CD3 Ab concentrations (3 µg/ml Ab, Figure 7b) indicating that the effects of NMDAR antagonism also depend on the strength of T-cell stimulation.

Consequently, I asked whether the addition of exogenous IL-2 or CD28 co-stimulation would abrogate or milden the inhibitory effects of NMDAR antagonists on T-cell proliferation. Indeed, in the presence of IL-2 or under CD28 co-stimulatory conditions NMDAR antagonist ifenprodil had a significant lower inhibitory effect on T-cell expansion compared to T cells stimulated with CD3 Abs only (Figure 7c). These results indicate that NMDAR-induced signals contribute to T-cell activation and that their inhibition impairs T-cell proliferation, especially under weak stimulatory conditions or in the absence of CD28 co-stimulation. This may result from an altered T-cell signalling and IL-2 production after antagonist addition.

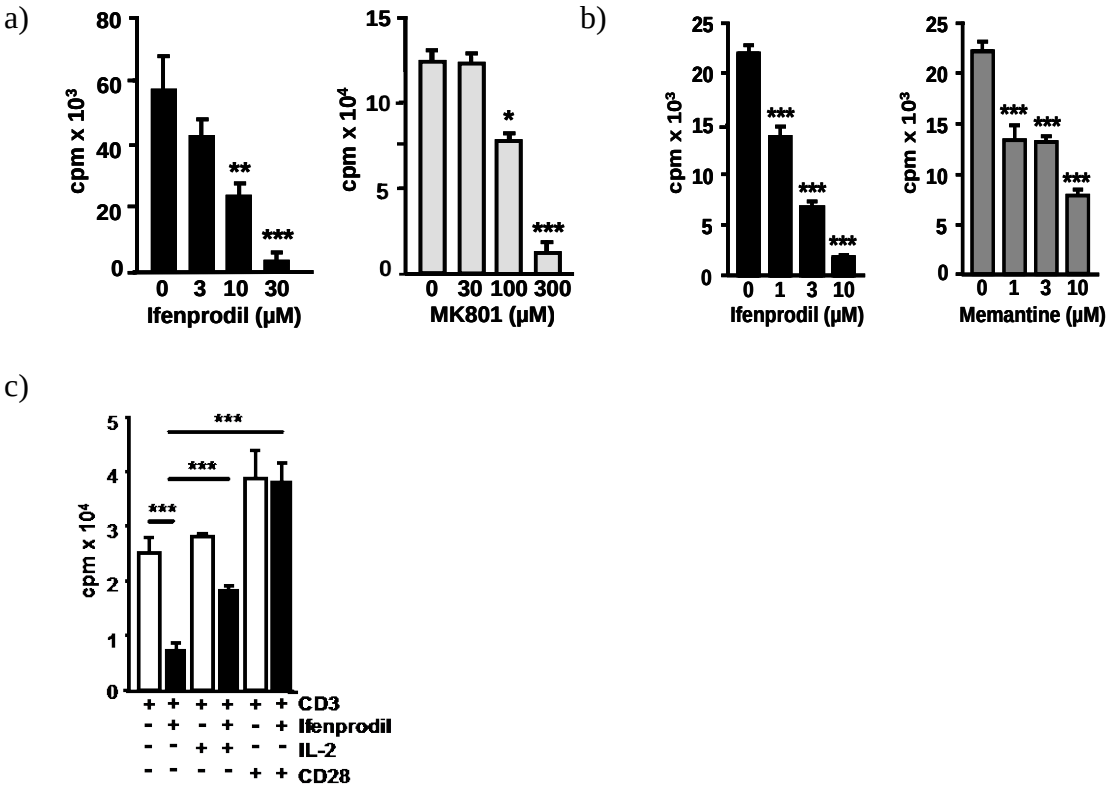


Figure 7. NMDAR antagonists block T-cell proliferation. CD4+ T cells were stimulated with **a)** CD3 Abs (10 µg/ml) in the presence or absence of ifenprodil and MK801 in the indicated concentrations, **b**) CD3 Abs (3 µg/ml) in the presence or absence of ifenprodil and memantine or **c)** CD3 Abs (3 µg/ml) or CD3+CD28 Abs (3 and 5 µg/ml) with or without ifenprodil (50 µM) and IL-2 (20 U/ml). Proliferation was determined by ^{3}[H]-Thymidine incorporation (cpm) at 24 h. Data show the mean + SD cpm of triplicates and are representative for 3 experiments. Significant *p* values were calculated by Student's *t* test with $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Kahlfuss et al., 2014).

3.1.3 GluN1 protein expression in murine T cells is elusive

Although mRNA for NMDAR subunits was detected by RT-PCR, apparent protein expression of NMDAR subunits by intracellular FACS staining, and NMDAR antagonists inhibited T-cell proliferation, we could not reliably detect surface expression of NMDAR subunits in murine thymocytes or peripheral T cells using two different GluN1 Abs detecting extracellular epitopes of GluN1 and standard flow cytometry. To clarify the missing evidence of NMDAR surface expression, protein expression of the obligatory GluN1 subunit was analysed by Western blot. GluN1 protein in thymocytes (Figure 8a) and CD4+ T cells (data not shown) appeared in three distinct immunoreactive bands, with the major band at ~110 kDa, i.e. at a lower MW than found for GluN1 from total brain cells with ~120 kDa. RT-PCR analyses for GluN1 mRNA revealed that the lower MW could result from the detected alternative splice forms of GluN1 in murine thymocytes (data not shown). Immunopreciptiation experiments to pull down GluN1 subunits from thymocytes or CD4+ T

cells were unsuccessful (data not shown). These negative results suggested that the GluN1 Abs may not detect GluN1 protein in thymocytes and CD4+ T cells and that the Abs used by us and others (Affaticati et al., 2011) might be specific for GluN1 in neuronal cells, but not for GuN1 in lymphocytes. To clarify this question, I analysed GluN1 knockout (ko) mice, which do not express NMDARs due to the loss of the obligatory GluN1 subunit. GluN1 ko mice were generated by crossing GluN1$^{flx/flx}$ mice with tg CMV-Cre deleter mice to delete GluN1 in all cells (Sprengel and Single, 1999). These mouse lines were kindly provided by Prof. R. Sprengel (MPI, Heidelberg). A feature of GluN1 deletion is that newborn mice die within hours after birth due to a critical defect of the central respiratory system and loss of the sucking reflex. To discriminate GluN1 wildtype (wt) or heterozygous (het) mice from GluN1 ko mice, newborns were monitored for 6 to 8 h after birth. Wt newborn mice would have sucked milk from the mother within that time, but not GluN1 ko newborns, allowing the identification of GluN1 ko newborns by the lack of milk in their stomach (Figure 8b).

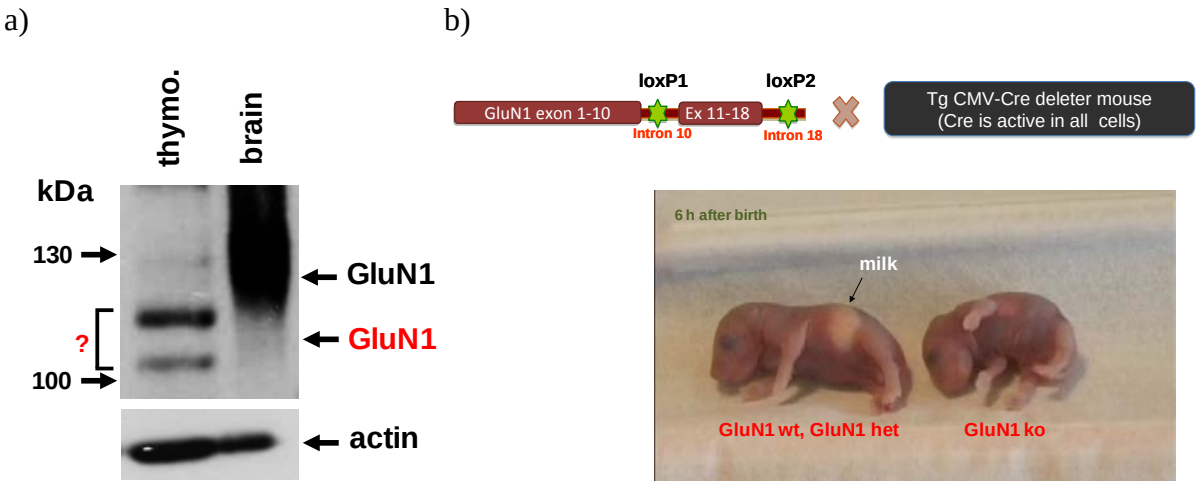


Figure 8. Analysis of GluN1 protein expression and generation of GluN1 ko mice. a) Western blot analysis of GluN1 protein expression (GluN1 Ab from Synaptic Systems) in protein extracts from thymocytes and total brain. Actin levels served as a protein loading control. MW markers are indicated. **b**) Strategy to generate GluN1 ko mice by crossing GluN1$^{flx/flx}$ with transgenic (Tg) CMV-Cre deleter mice. LoxP sites for the excision of exons 11-18 of GluN1 are marked. The photo shows newborn mice 6 h after birth. The GluN1 wt/het but not the GluN1 ko mouse shows milk in the stomach.

Besides initial discrimination by stomach milk content, DNA was isolated from GluN1 wt/het and GluN1 ko thymocytes and PCR for the floxed GluN1 alleles and the excision of exons 11-18 of GluN1 was performed (Figure 9a). In addition, mRNA was isolated from the respective thymocytes to run RT-PCRs for GluN1 mRNA expression (Figure 9b). In GluN1

ko newborns GluN1 deletion was detected at DNA and mRNA level and thus they should not express GluN1 protein in thymocytes.

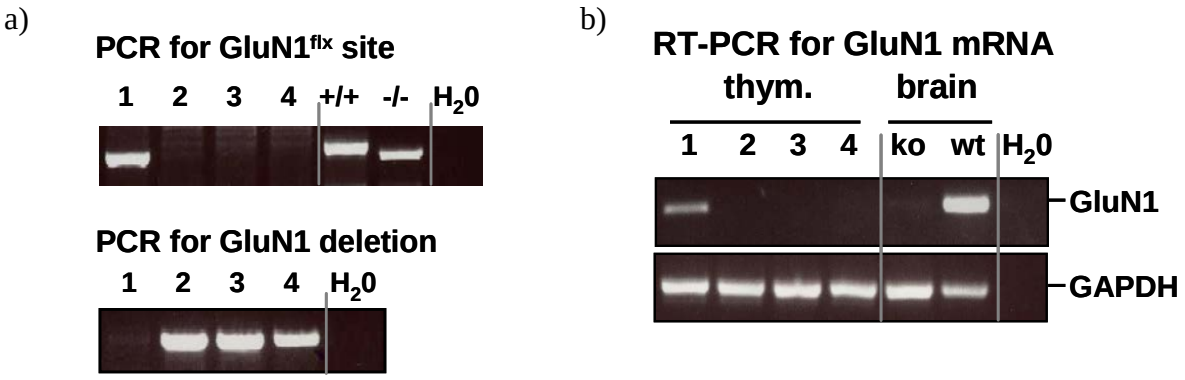


Figure 9. Detection of GluN1 deletion in thymocytes. DNA and mRNA were isolated from thymocytes and brains of newborn GluN1 ko littermate mice and analysed for GluN1. **(a)** PCR analysis of thymocyte DNA for floxed (+) and wt (-) GluN1 alleles (upper panel) and excision of the floxed GluN1 sequence (lower panel). **(b)** RT-PCR analysis of GluN1 and GAPDH mRNA expression in thymocytes and brains of wt and GluN1 ko mice. In (a) and (b), lane 1 represents thymocytes from a GluN1 wt mouse and lanes 2-4 thymocytes from GluN1 ko mice (Kahlfuss et al., 2014).

Protein extracts from thymocytes and the corresponding brains of PCR-typed GluN1 ko and wt newborn mice were further used for Western blot analysis. As shown in Figure 10a, GluN1 protein (~120 kDa) was deleted in brain samples of GluN1 ko mice (Figure10a, brain, lanes 4 and 5) compared to GluN1 wt mice (Figure10a, brain, lane 6). However, in thymocyte samples of the same mice the routinely observed three bands for GluN1 protein in GluN1 wt/het mice (Figure10a, thymocytes, lanes 1-3 and 6) were still present in GluN1 ko mice (Figure10a, thymocytes, lanes 4 and 5). These data strongly suggest that the used GluN1 Ab does not detect GluN1 protein in thymocytes but rather cross-reacts with some unknown protein. In addition, intracellular FACS staining with two different GluN1 Abs showed an identical GluN1 staining in wt and ko thymocytes (Figure 10b, data provided by S. Kahlfuß, PhD thesis 2015 (Kahlfuss et al., 2014)). The latter results further support the conclusion that the used GluN1 Abs show an unspecific staining in thymocytes.

Hence, on the protein level, there is no evidence for the expression of the obligatory GluN1 subunit of NMDARs in murine thymocytes and peripheral T cells.

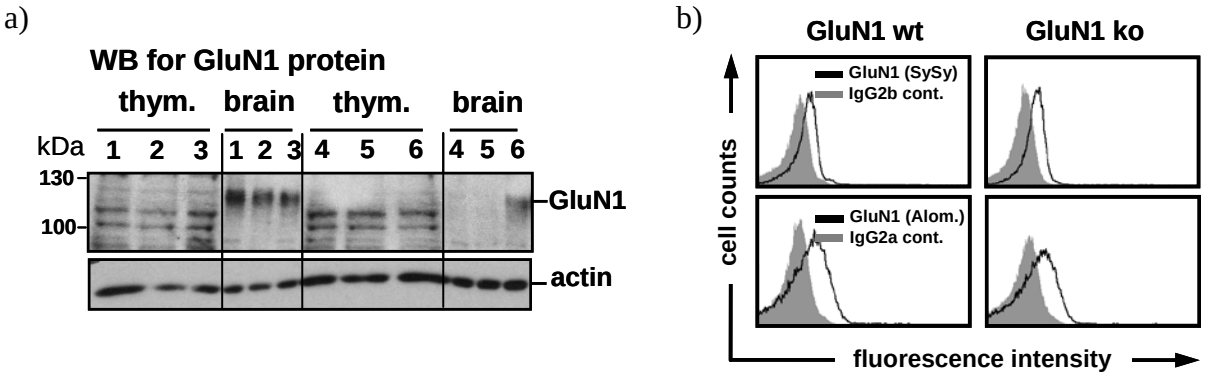


Figure 10. Assessment of GluN1 protein expression in GluN1 ko and wt mice. a) Western blot analysis was performed for GluN1 protein (GluN1 Ab from Synaptic Systems) in thymocytes and brains of GluN1 wt and ko mice. Actin expression served as a control for protein loading. Lanes 4 and 5 represent GluN1 ko mice, lanes 1-3 GluN1 het mice and lane 6 shows a GluN1 wt mouse. **(b)** Thymocytes from GluN1 wt and GluN1 ko mice were analysed for GluN1 protein expression by intracellular FACS staining and flow cytometry. GluN1 Abs were from Synaptic Systems (SySy) or Alomone Labs (Alom.). Shaded histograms represent isotype control stainings. Data in b) were kindly provided by S. Kahlfuss (Kahlfuss, 2015; Kahlfuss et al., 2014).

Altogether, the results allow the conclusion that NMDARs are either expressed in very low numbers and only transiently come to the cell surface such that the detection methods used by us and others are inadequate to conclude their expression in T cells. As mentioned in the publication of the Affaticati group (Affaticati et al., 2011), we also could not detect surface expression of NMDARs on T cells using whole cell patch clamp analyses (personal communication by Dr. T. Bose, LIN, Magdeburg). Thus, the results eventually obtained from the analyses of GuN1 ko mice show that protein expression of NMDARs in murine T lymphocytes is elusive, if not absent. In view of the obvious effects of NMDAR antagonists on T-cell proliferation, we hypothesized that the antagonists cross-inhibit other ion channels expressed on lymphocytes.

To further substansiate this, an important experiment was to determine the proliferation of GluN1 ko thymocytes in the presence of NMDAR antagonists (Figure 11). Thymocytes were isolated from GluN1 wt and ko newborn mice and stimulated with CD3 Abs (3 μg/ml) in the absence or presence of different concentrations of ifenprodil. The proliferation of GluN1 ko thymocytes was similar to wt thymocytes in the absence of ifenprodil. Notably, ifenprodil inhibited the proliferation of GluN1 ko thymocytes to a similar degree as found for wt thymocytes. These results led us to conclude that NMDAR antagonists affect thymocytes and T cells not via specific inhibition of NMDARs, but rather by cross-inhibition of other targets.

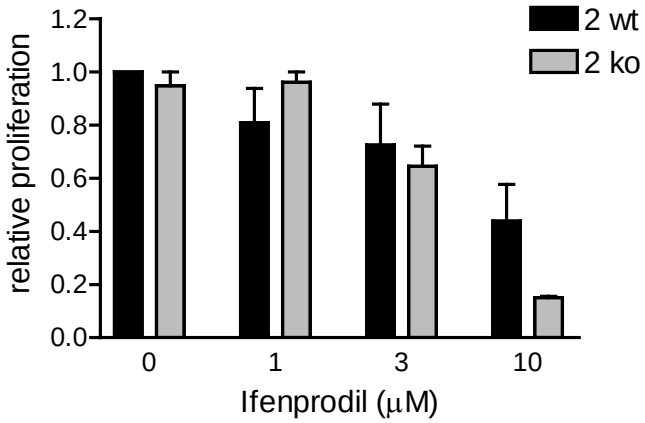


Figure 11. Ifenprodil inhibits the proliferation of GluN1 ko thymocytes. Thymocytes isolated from newborn littermate GluN1 ko and GluN1 wt mice (two mice each) were stimulated in triplicates with CD3 Abs (3 µg/ml) in the presence of the indicated concentrations ifenprodil. Proliferation was determined by ^{3}[H]-Thymidine incorporation at 24 h and is shown as mean + SD relative proliferation. Similar results were obtained in 3 experiments.

Indeed, within our collaborative research project my colleague T. Bose showed in her PhD work that the used NMDAR antagonists ifenprodil and memantine cross-inhibit K_v1.3 and K_{Ca}3.1 potassium channels expressed on T cells and lead to a decrease of Ca^{2+}-flux upon TCR stimulation (Kahlfuss et al., 2014; T. Bose, PhD thesis, 2014). For further understanding and discussion, some of her patch clamp results are shown in Figure 12.

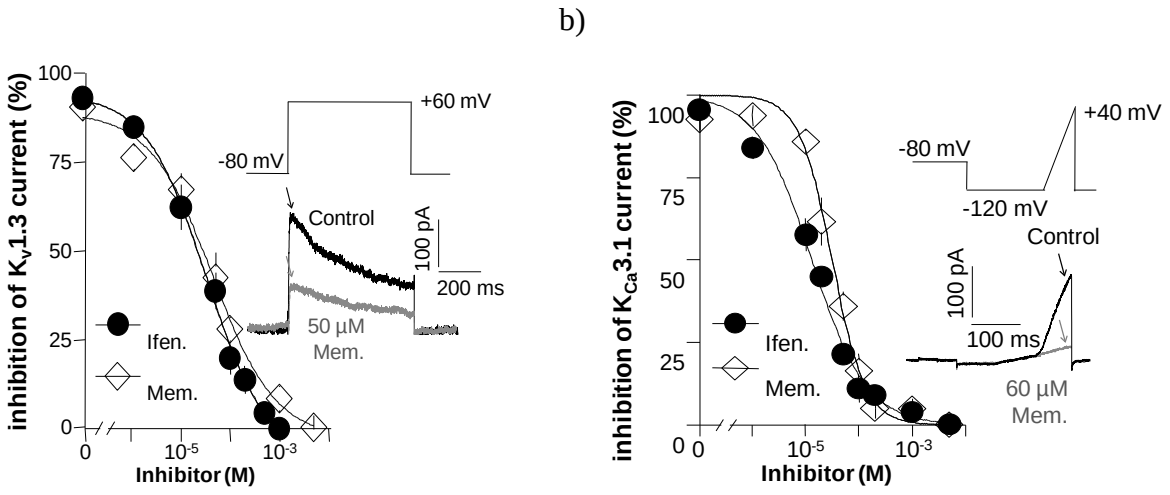


Figure 12. NMDAR antagonists cross-inhibit K_v1.3 and K_{Ca}3.1 potassium channels. $CD4^+$ T cells were activated with CD3+CD28 Abs (3+5 µg/ml) in the presence of the NMDAR antagonists ifenprodil (Ifen.) or memantine (Mem.) and the dose-response relationships for **(a)** K_v1.3 and **(b)** K_{Ca}3.1 channel-mediated currents were determined by whole cell patch-clamp. Inserts show voltage ramp protocols and examples of current traces for control conditions and in the presence of memantine. Data are normalized to the current measured under control conditions and represented as relative inhibition. Data points in the dose-response relationships represent mean values ± SD calculated from 5-7 cells each. Data were kindly provided by Dr. T. Bose (Kahlfuss et al., 2014).

3.1.4 NMDAR antagonist ifenprodil attenuates TCR-induced signalling

In the second part of my thesis, I investigated major signalling pathways in antagonist-treated lymphocytes in order to gain further understanding of the molecular mechanisms underlying the drugs' effects on T-cell activation. Isolated naive CD4+ T cells were stimulated with plate-bound CD3 Abs (10 μg/ml) in the presence or absence of ifenprodil (50 μM) for 0, 30, 60, 120, and 240 min. Protein extracts were analysed by Western blot for the activation of the Src kinases Lck/Fyn, and serine/threonine kinases Akt and Erk1/2. These short-term stimulation assays revealed that ifenprodil impairs the activation of Lck/Fyn, Erk1/2 as well as Akt (Figure 13).

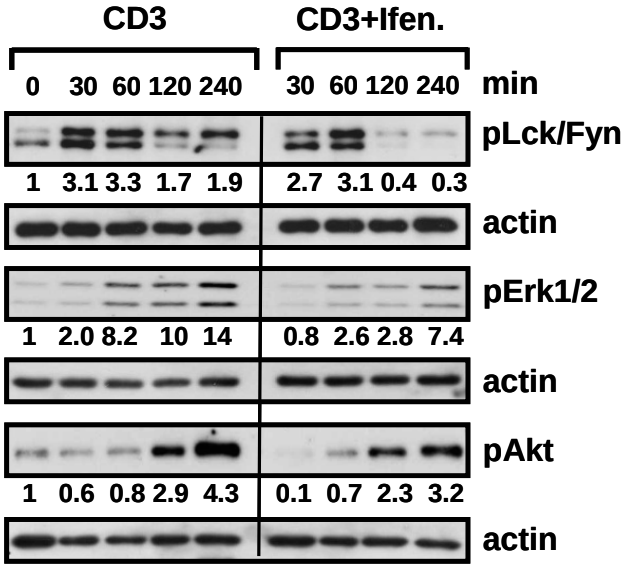


Figure 13. NMDAR antagonist ifenprodil attenuates TCR signalling. CD4+ T cells were stimulated with plate-bound CD3 Abs (10 μg/ml) in the presence or absence of ifenprodil (50 μM) for 0, 30, 60, 120, and 240 min. Total protein extracts were analysed for the activation of the indicated signalling molecules using p-specific Abs and Western blot analysis. Actin expression served as a control for protein loading. The indicated numbers give the relative protein expression after quantification and normalization to the respective actin controls. Data are the representative of 3 independent experiments (Kahlfuss et al., 2014).

As I found that major signalling events in short-term TCR-activated CD4+ T cells were attenuated by NMDAR antagonist ifenprodil, it was essential to investigate whether the inhibitor would also influence signalling events at later time points. CD4+ T cells were stimulated with plate-bound CD3 Abs (3 μg/ml) in the presence or absence of ifenprodil (30 μM) for 0, 8, 16, and 24 h followed by the analysis of cytoplasmic and nuclear protein extracts for the the phosphorylation status of PLCγ1, GSK3β, mTOR, and S6 by Western blot. In the presence of ifenprodil, the levels of cytoplasmic pPLCγ1, pGSK3β, pmTOR, and

pS6 were significantly reduced compared to untreated cells (Figure 14). These results indicate a lower or, in the case of GSK3β, an enhanced activity of these signalling molecules during later phases of T-cell activation and thus a long-lasting effect of ifenprodil on PLCγ1- as well as Akt-induced signalling events.

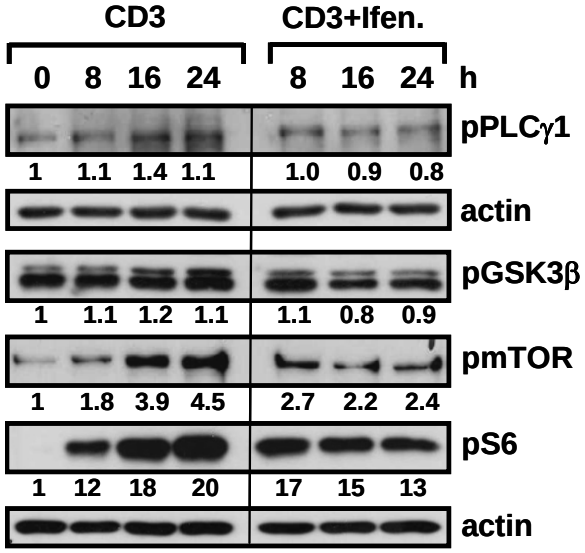


Figure 14. NMDAR antagonist ifenprodil attenuates signalling in long-term stimulated T cells. CD4+ T cells were stimulated with plate-bound CD3 Abs (3 µg/ml) in the presence or absence of ifenprodil (30 µM) for the indicated time points and cytoplasmic protein extracts were analysed for the phosphorylation of PLCγ1, GSK3β, mTOR, and S6 by Western blot. Actin expression served as a protein loading control. Indicated numbers give the relative protein expression after quantification and normalization to the actin controls. Data are the representative of 3 independent experiments (Kahlfuss et al., 2014).

Several serine protein kinases, including GSK3β, intracellular Ca^{2+}-level and the phosphatase calcineurin control NFAT activation. Inactive cytoplasmic NFAT is dephosphorylated by calcineurin leading to nuclear localization of NFAT and, among other genes, to the transcriptional induction of *NFATc1* (Chuvpilo et al., 2002; Hogan et al., 2010). In view that NMDAR antagonists reduce TCR-induced Ca^{2+}-mobilization (T. Bose, PhD thesis 2014, (Kahlfuss et al., 2014)), it was interesting to determine to what extent ifenprodil impacts on NFATc1 activation in long-term stimulated CD4+ T cells. As shown in Figure 15, CD4+ T cells stimulated for 24 h in the presence of ifenprodil had a 65% reduction in nuclear NFATc1 in comparison to cells cultured without drug. Thus, ifenprodil interferes with the maintenance of NFATc1 activation, which may contribute to the drug-induced inhibition of proliferation.

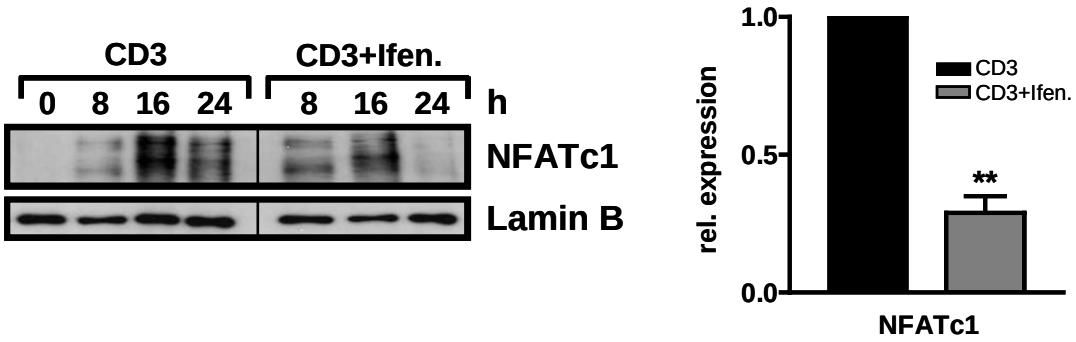


Figure 15. Ifenprodil reduces nuclear NFATc1 accumulation. Nuclear protein extracts of CD4+ T cells stimulated with CD3 Abs (3 µg/ml) for 8, 16 and 24 h in the presence or absence of ifenprodil (30 µM) were analysed for NFATc1 expression by Western blot. Lamin B served as a protein loading control. Data in the right graph represent the mean relative +SD NFATc1 expression after densitometric quantification and relation to Lamin B controls. Data were calculated from 3 independent experiments. Significance was calculated by Student's *t* test with $**p < 0.01$ (Kahlfuss et al., 2014).

NMDAR antagonist ifenprodil had less inhibitory effects on proliferation when CD4+ T cells were co-stimulated with CD3+CD28 Abs (Figure 7c). Thus, I compared the signalling events in CD4+ T cells stimulated for 24 h with CD3 Abs alone (3 µg/ml) or with CD3+CD28 Abs (3+5 µg/ml) in the presence or absence of ifenprodil (30 µM) (Figure 16). Ifenprodil treatment led to a reduced phosphorylation of PLCγ1, GSK3β, mTOR, and S6 in co-stimulated CD4+ T cells similar to that in CD3 Ab-stimulated cells.

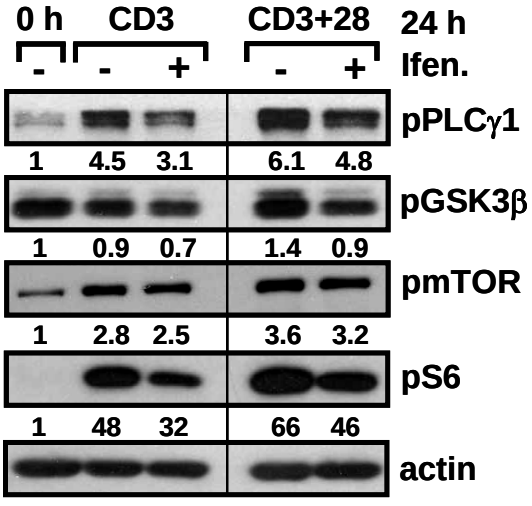


Figure 16. CD28 signals rescue the inhibitory effects of ifenprodil on TCR-induced signalling. CD4+ T cells were stimulated with plate-bound CD3 Abs (3 µg/ml) or CD3+CD28 Abs (3+5 µg/ml) in the presence or absence of ifenprodil (30 µM) for 24 h and cytoplasmic protein extracts were analysed for the indicated proteins by Western blot. Actin expression served as a control for protein loading. Indicated numbers give the relative protein expression after quantification and normalization to the actin control. Data are the representative of 2 independent experiments (Kahlfuss et al., 2014).

However, the level of phosphorylated proteins in co-stimulated T cells was still higher than after CD3 stimulation alone and corresponded to that found in CD3 Ab-activated T cells cultured without ifenprodil (Figure 16).

Altogether, the obtained data show that NMDAR antagonist ifenprodil impairs T-cell activation by attenuating important TCR-induced signalling events, including the activation of Lck/Fyn, PLCγ1, Erk1/2, Akt, S6, and NFATc1. The drug's inhibitory effect, at least partially, is overcome by CD28 signals.

3.2 The effects of NMDAR antagonists on B cells

B cells are important effectors and regulators of adaptive and innate immune responses by providing antigen presentation and co-stimulation for CD4+ T cells, secreting cytokines, and production of antigen-specific Abs. By formation of auto-Abs B cells can also mediate destructive immune responses as in autoimmune neuroinflammation (Bittner and Meuth, 2013; Lueg et al., 2015).

As NMDAR antagonist memantine is in clinical use, it is important to understand the drug's possible side-effects on B cells. Thus, I investigated whether NMDARs are expressed in B cells and how NMDAR antagonists affect B-cell signalling, proliferation, IL-10 production, and IgM/IgG production. Since B cells can be activated via ligation of the BCR or by LPS/TLR4, I used both stimulatory conditions for most experiments.

3.2.1 Detection of NMDAR subunits in B cells

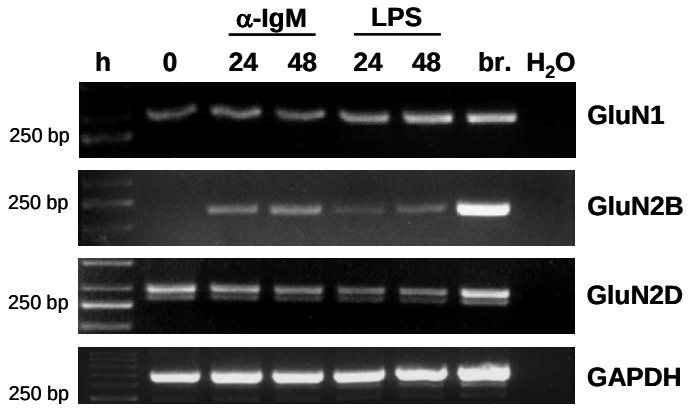


Figure 17. RT-PCR analysis detects mRNA of NMDAR subunits in B cells. mRNA expression of GluN1, GluN2B and GluN2D subunits in B cells activated with α-IgM (10 µg/ml) or LPS (10 µg/ml) for 24 and 48 h and in brain was determined by RT-PCR. GAPDH mRNA expression was used as RT-PCR control. Data are the representative of 2 experiments.

First, B cells were stimulated with α-IgM or LPS for 24 and 48 h and mRNA was isolated from the activated B cells. RT-PCR revealed mRNA expression for GluN1, GluN2B and GluN2D subunits (Figure 17) suggesting the expression of NMDARs in B cells. However, as outlined before for T cells, a reliable detection of GluN1 protein in B cells by Western blot was not feasible (data not shown).

3.2.2 NMDAR antagonists block BCR- and LPS-induced B-cell proliferation

$K_v1.3$ channels are expressed on B cells and their inhibition was shown to reduce BCR- and PMA/IO-induced B-cell proliferation (Amigorena et al., 1990; Partiseti et al., 1992; Wulff et al., 2004). Thus, we suspected that NMDAR antagonists could also affect B cells via cross-inhibition of $K_v1.3$ and $K_{Ca}3.1$ channels.

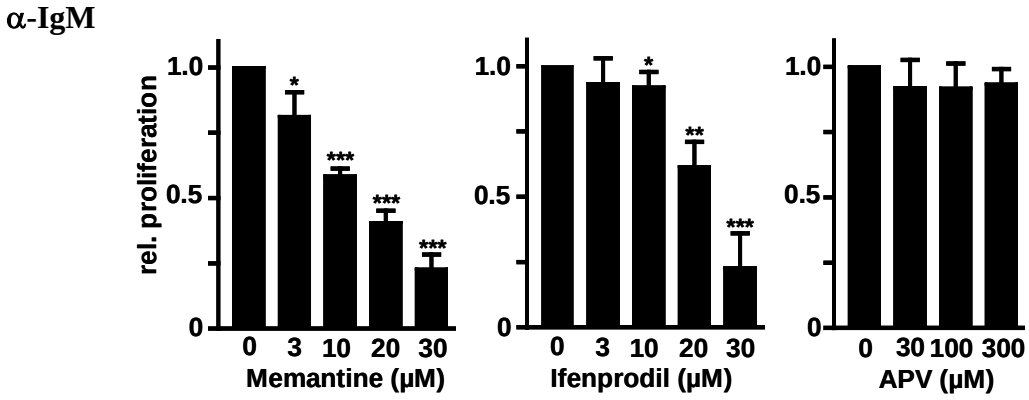


Figure 18. NMDAR antagonists inhibit α-IgM-induced B-cell proliferation. Splenic B cells were stimulated with α-IgM (10 μg/ml) in the presence or absence of memantine, ifenprodil and D-APV in concentrations as indicated. Proliferation was determined by ^{3}[H]-Thymidine incorporation (cpm) at 24 h. Data in the graphs represent the mean + SD relative proliferation of at least 3 experiments. Significant *p* values were calculated with Student's *t* test with *p<0.05, **p<0.01 and ***p<0.001 (Simma et al., 2014).

Indeed, my collaborator Dr. T. Bose showed that ifenprodil and memantine, but not D-APV, significantly reduce $K_v1.3$ channel currents on B cells stimulated with α-IgM or LPS. Reduced $K_{Ca}3.1$ channel currents were detected only in BCR- but not LPS-activated B cells. (data not shown, T. Bose, PhD thesis 2014, (Simma et al., 2014)). Thus, the major targets of ifenprodil or memantine in B cells most likely are not NMDARs but $K_v1.3$ and $K_{Ca}3.1$ channels.

First, I investigated the role of NMDARs on B-cell proliferation. Isolated splenic B cells were stimulated with IgM F(ab')$_2$ Abs (α-IgM) (10 μg/ml) to mimic BCR triggering by antigens or with the TLR4 ligand LPS (10 μg/ml) in the presence of memantine, ifenprodil or the

competitive antagonist D-APV. B-cell proliferation was determined by ^{3}[H]-Thymidine incorporation at 24 h. As shown in Figure 18 and Figure 19, memantine and ifenprodil significantly inhibited α-IgM- as well as LPS-induced B-cell proliferation in a concentration dependent manner. In contrast, even the highest concentrations of D-APV (300 μM) had no effect on B-cell proliferation. The latter underlines that the anagonists do not act on NMDARs, but through inhibition of other channels.

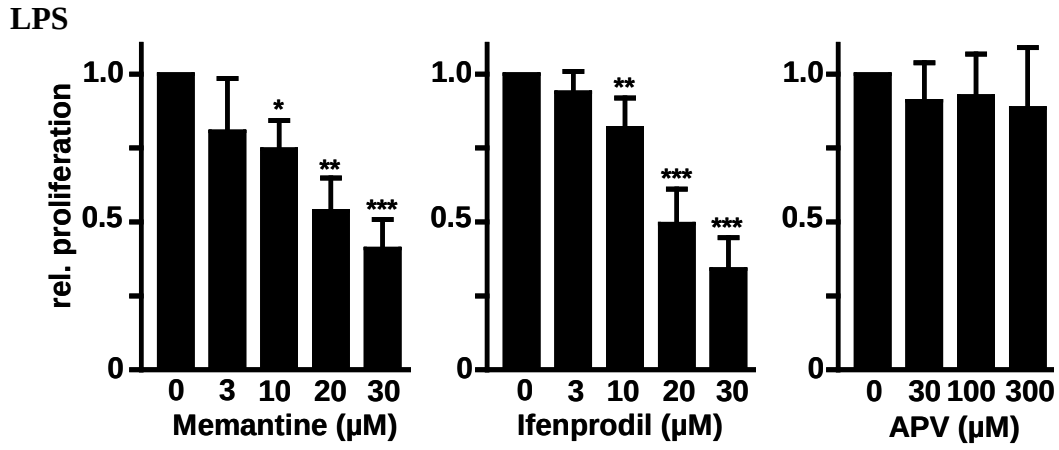


Figure 19. NMDAR antagonists inhibit LPS-induced B-cell proliferation. Splenic B cells were stimulated with LPS (10 μg/ml) in the presence or absence of memantine, ifenprodil and D-APV in concentrations as indicated. Proliferation was determined by ^{3}[H]-Thymidine incorporation (cpm) at 24 h. Data in the graphs represent the mean + SD relative proliferation of at least three experiments. Significant *p* values were calculated with Student's *t* test with *p<0.05, **p<0.01 and ***p<0.001 (Simma et al., 2014).

3.2.3 NMDAR antagonists attenuate BCR- as well as LPS-induced B-cell signalling

Since the antagonists inhibited B-cell proliferation, I addressed how they influence BCR- and LPS-induced signalling events. B cells were stimulated with α-IgM (10 μg/ml) or LPS (10 μg/ml) in the presence or absence of ifenprodil (30 μM) for 1, 5, 10, 30, and 60 min followed by Western blot analysis for the expression of pAkt, pS6 and pErk1/2. Compared to untreated cells, the presence of ifenprodil led to a reduced activation of these signalling molecules (Figure 20, left panel). Interestingly, LPS/TLR4-stimulated B cells showed a very similar inhibition of Akt, S6 and Erk1/2 phosphorylation in the presence of ifenprodil (Figure 20, right panel). Further experiments investigated the effects of NMDAR antagonists on B-cell signalling events at later time points. For this, B cells were activated with α-IgM (10 μg/ml) or LPS (10 μg/ml) in the presence or absence of ifenprodil (30 μM) for 12 and 24 h (Figure 21).

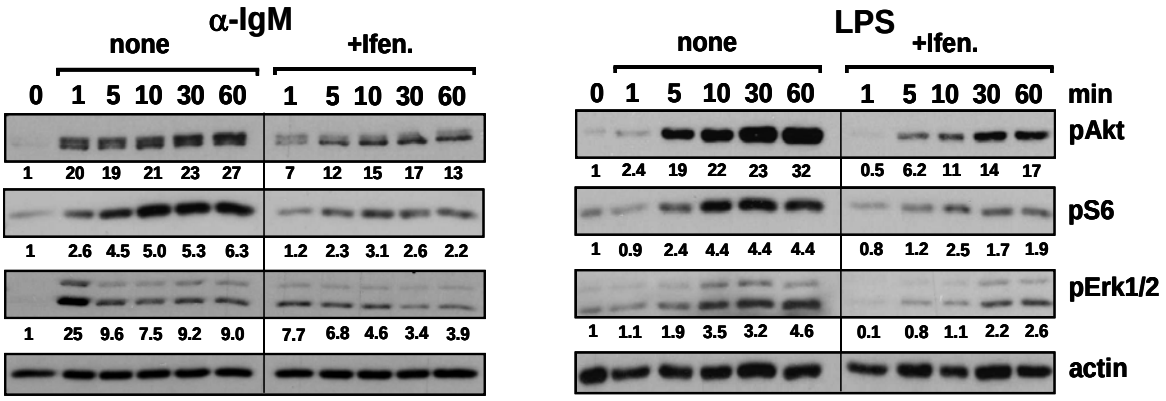


Figure 20. NMDAR antagonist ifenprodil attenuates BCR- and LPS-induced signalling events in short-term B-cell stimulation. Splenic B cells were activated with α-IgM or LPS (10 µg/ml) in the presence or absence of ifenprodil (30 µM) for 1, 5, 10, 30, and 60 min and total protein extracts were analysed for the indicated signalling molecules by Western blot. Actin expression served as a loading control. Indicated numbers give the relative protein expression after quantification and normalization to actin controls. Data are the representative of 3 independent experiments (Simma et al., 2014).

In cytoplasmic protein extracts of ifenprodil-treated cells, the phosphorylation of S6 and Erk1/2 was reduced at 12 and 24 h compared to untreated cells (Figure 21a). In addition, the nuclear accumulation of pErk1/2 and NFATc1 was diminished (Figure 21b). Thus, NMDAR antagonist ifenprodil downregulates major signalling events of two distinct B-cell activating receptors that play an important role in innate and antigen-specific B-cell responses (Bhattacharyya et al., 2011; Hock et al., 2013).

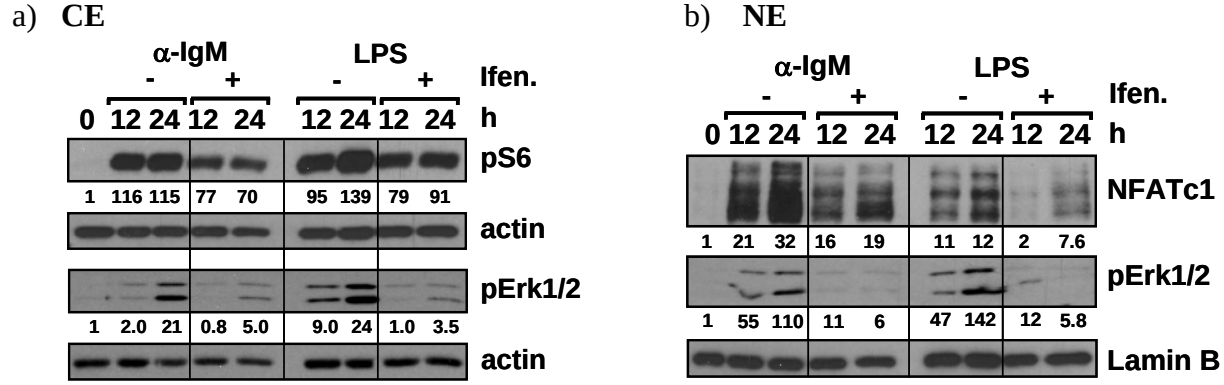


Figure 21. Effect of ifenprodil on BCR- and LPS-induced signalling molecules in long-term stimulation. Splenic B cells were activated with α-IgM or LPS (10 µg/ml) in the presence or absence of ifenprodil (30 µM) for 12 and 24 h and **(a)** cytoplasmic (CE) and **(b)** nuclear (NE) protein extracts were analysed for the indicated proteins by Western blot. Actin (a) and Lamin B (b) served as protein loading controls. Indicated numbers give the relative protein expression after quantification and normalization to the controls. Data are the representative of 3 independent experiments (Simma et al., 2014).

3.2.4 CD40 co-stimulation maintains BCR signalling in the presence of ifenprodil

Additionally, I investigated the effect of ifenprodil on signalling events in BCR+CD40 co-stimulated B cells as we had found that CD40 co-stimulation rescues the antagonist-provoked inhibition of BCR-induced B-cell proliferation (Simma et al., 2014). B cells were stimulated in the absence or presence of ifenprodil (30 μM) for 12 and 24 h. Drug treatment reduced the activation of Erk1/2 and S6 in α-IgM+CD40-activated B cells. However, the level of phosphorylation of Erk1/2 and S6 was still markedly higher than that found in B cells treated with α-IgM plus ifenprodil and similar to the activation found in α-IgM-activated B cells in the absence of ifenprodil (Figure 22). These results strongly suggest that the drug-induced attenuation of signalling in CD40 co-stimulated B cells still remains above the critical threshold needed for B-cell activation.

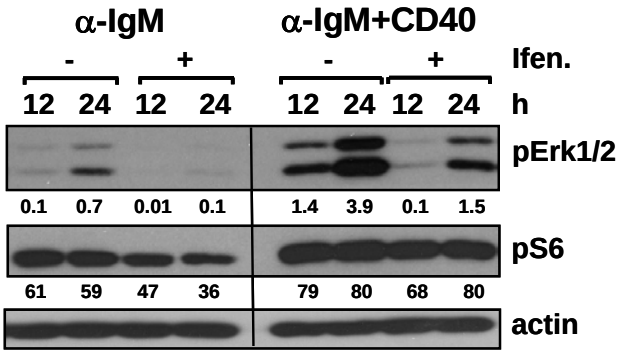


Figure 22. CD40 co-stimulation maintains strong signalling in the presence of ifenprodil. Splenic B cells were activated with α-IgM (10 μg/ml) and CD40 (5 μg/ml) in the presence or absence of ifenprodil (30 μM) for 12 and 24 h and cytoplasmic protein extracts were analysed for the indicated signalling molecules by Western blot. Actin served as a protein loading control. Indicated numbers give the relative protein expression after quantification and normalization to the actin controls. Data are the representative of 2 independent experiments (Simma et al., 2014).

3.2.5 Ifenprodil modulates IL-10 production of B cells

B10 cells, a small subpopulation of B cells, exert an important immunoregulatory role by producing the immunosuppressive cytokine IL-10 (Bhattacharyya et al., 2011; Fillatreau et al., 2002; Mauri et al., 2003; Yoshizaki et al., 2012). Next, I asked whether the drug-induced attenuated B-cell signalling would influence the production of IL-10. B cells were stimulated with the mitogens PMA and IO in the presence or absence of ifenprodil for 16 h and IL-10 mRNA expression was assessed by RT-PCR. IL-10 mRNA was induced upon B-cell stimulation. However, additional co-treatment of B cells with ifenprodil resulted in a marked repression of IL-10 mRNA compared to untreated B cells (Figure 23a).

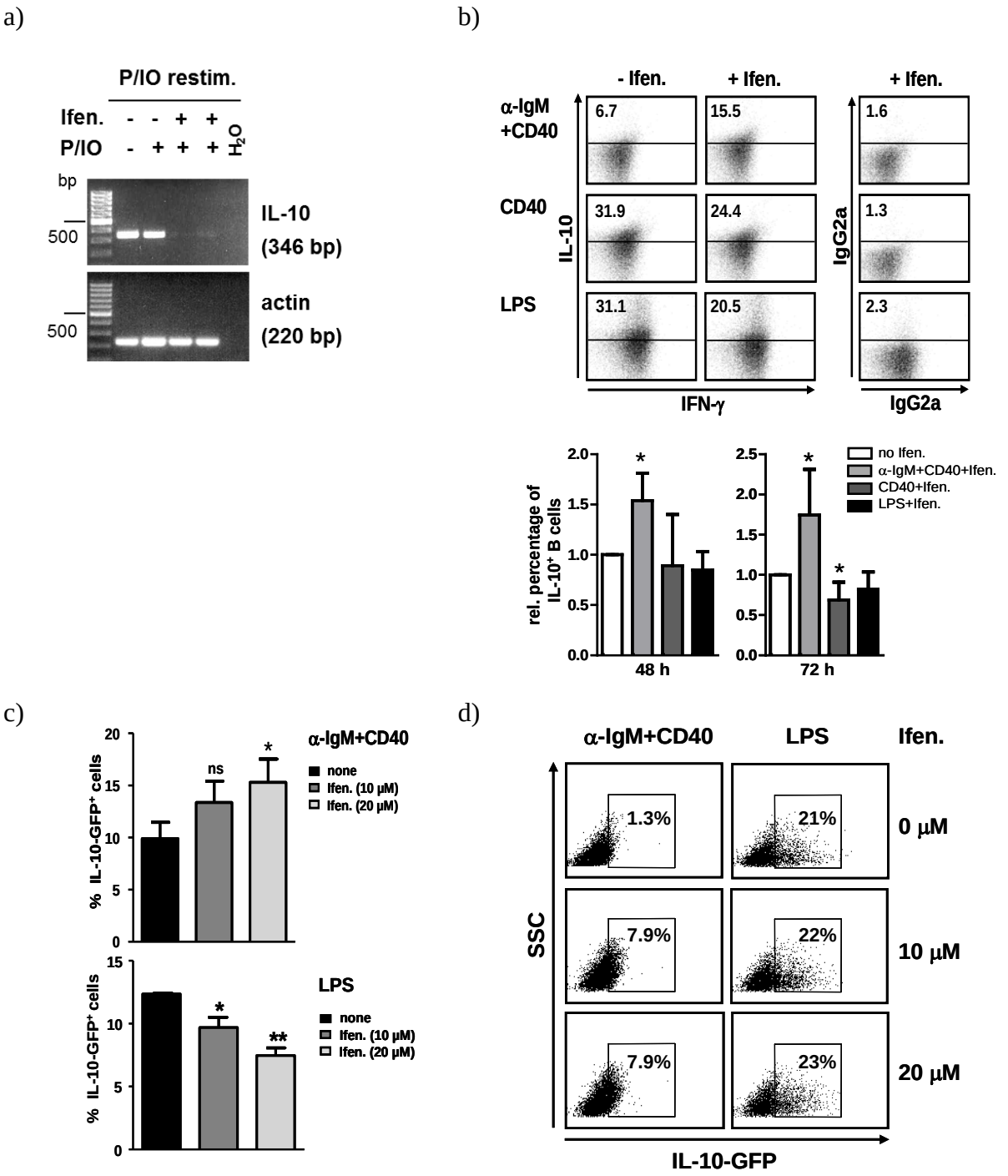


Figure 23. NMDAR antagonists modulate B cell IL-10 production. a) Splenic B cells were left untreated (lane 1) or were activated with PMA/IO in the absence (lane 2) or presence of ifenprodil (10 and 30 μM, lanes 3 and 4) for 16 h. Cells were re-stimulated with PMA/IO (in the presence of monensin) for 6 h before cell harvest. RT-PCR analysis was used to determine IL-10 and actin mRNA expression. Data are the representative of 2 experiments. **b)** B cells were stimulated for 2-3 days with either α-IgM+CD40 Abs, CD40 Abs alone or with LPS. Ifenprodil (10 μM) was given at day 1. After cell harvest, intracellular expression of IL-10 and IFN-γ was determined by FACS staining and flow cytometry. Data are the representative of 2-4 experiments. **c, d)** B cells from IL-10-GFP tiger mice were activated with α-IgM+CD40 Abs or LPS. Ifenprodil (10 or 20 μM) was added after 21-25 h and the percentage of cells expressing IL-10-GFP was determined at c) day 2 or d) day 4 with flow cytometry. The data in the graphs were caluclated from 2 independent experiments with *p<0.05 and **p<0.01 (Simma et al., 2014).

In addition, with intracellular IL-10 staining and flow cytomety it was determined how ifenprodil affects IL-10 production when added at later time points, i.e. 1 day after B cells had been activated with α-IgM+CD40, CD40 Abs alone or LPS (Figure 23b).

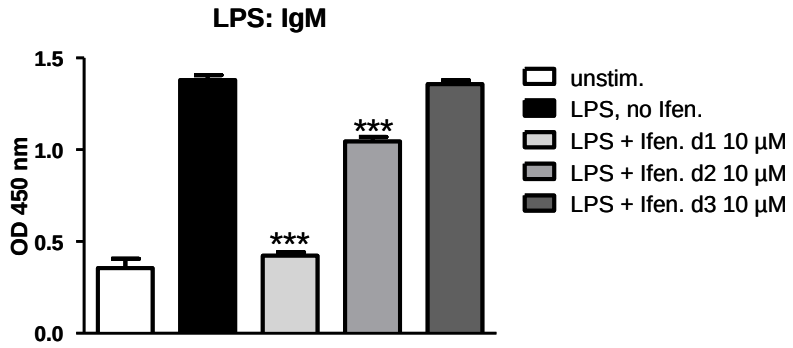
CD40 and LPS are known to give rise to regulatory B10 cells. Consequently, we found that 19-27% of B cells produced IL-10 after CD40 or LPS stimulation, whereas only 8% of B cells were positive for IL-10 after α-IgM+CD40 activation. In LPS- or CD40-treated cultures the percentage of IL-10 producers was not substantially altered by ifenprodil, or even reduced. However, ifenprodil significantly increased the percentage of IL-10 producers in α-IgM+CD40-stimulated B cells by 1.5-2.0- fold. There was no effect of ifenprodil on IFN-γ production (Figure 23b).

To confirm above results, B cells from IL-10-GFP knock-in tiger mice were analysed, allowing visualization of IL-10 via its GFP (green fluorescent protein)-tag and without intracellular Ab staining. Splenic B cells from IL-10-GFP tiger mice were stimulated with α-IgM+CD40 or LPS and ifenprodil (10 or 20 μM) was added to the cultures after 21-25 h (Figure 23c, d). A 50% increase in the frequeny of IL-10-GFP producing B cells was found at day 2 after α-IgM+CD40 Ab stimulation (Figure 23c) and a 6-fold increase at day 4 (Figure 23d) when ifenprodil was present in the cultures. The frequency of LPS-induced IL-10 producing B cells was either reduced or ifenprodil had no major effect on their generation. Thus, ifenprodil can foster the generation of B10 cells.

3.2.6 Ifenprodil impairs IgM and IgG production of LPS-activated B cells

The major effector function of activated B cells is to produce Abs. Thus, I also assessed the impact of NMDAR antagonists on IgM and IgG secretion. B cells were stimulated with LPS (10 μg/ml) or LPS plus IL-4. Ifenprodil was added at day 1, 2 or 3 and culture supernatants were taken at day 4. The secreted IgM and IgG Abs were determined with ELISA (ELISA was performed by our collaborator Dr. F. Lühder, Göttingen). When ifenprodil (10 μM) was added at day 1, IgM and IgG production was almost totally abolished, and when added at day 2 IgM/IgG levels were still significantly decreased (Figure 24). Hence, in addition to B-cell proliferation, NMDAR antagonist ifenprodil has a profound inhibitory effect on IgM and IgG formation.

a)



b)

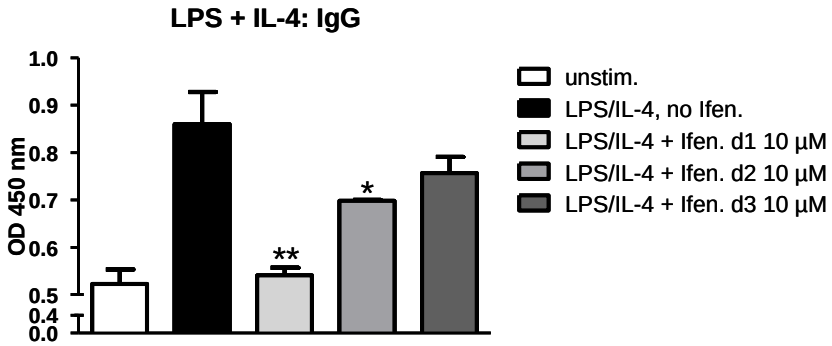


Figure 24. Ifenprodil blocks IgM/IgG production of LPS-stimulated B cells. Splenic B cells were activated with LPS (10 µg/ml) or LPS plus IL-4 (20 ng/ml). Ifenprodil (10 µM) was added at day 1, 2 or day 3. Harvested culture supernatants, taken at day 4, were analysed for IgM and IgG secretion by ELISA. The data in the graphs represent the mean + SD values at OD450 nm, with *p<0.05, **p<0.01 and ***p<0.001. Data are representative for 2 experiments (Simma et al., 2014).

In conclusion, NMDAR antagonists attenuate B-cell signalling, proliferation and block Ab production, but they can enhance B-cell IL-10 induction. Thus, NMDAR antagonists exert potent immunosuppressive and immunoregulatory effects not only on T cells, but also on B cells.

4. Discussion

4.1 The puzzle of NMDAR expression in lymphocytes

The expression of NMDAR subunits in human T cells and their effects on T-cell function, like adhesion and proliferation, had been reported in several publications and for murine thymocytes a role of NMDARs in Ca^{2+}-mobilization and apoptosis of DP cells was shown (Affaticati et al., 2011; Boldyrev, 2005; Levite, 2008; Pacheco et al., 2007). Using RT-PCR and intracellular FACS staining, we seemingly also detected the expression of GluN1 and GluN2A/B subunits in resting and activated murine CD4+ T cells. However, using two different commercial Abs, both detecting extracellular epitopes of the obligatory subunit GluN1, a surface expression of GluN1 on thymocytes and peripheral resting or activated T cells was not reliably detected using routine FACS staining and flow cytometry (Kahlfuss et al., 2014). In addition, in immunoprecipitation assays (data not shown) and Western blot analyses the detection of GluN1 protein was not feasible. This became most evident when I compared GluN1 expression in thymocytes and brains from GluN1 ko and wildtype mice. These analyses led us to conclude that the Abs, used by us and previously by Affaticati et al., are unsuitable for the detection of GluN1 in murine lymphocytes. Thus, at the protein level it is still unclear whether and to what extent NMDARs are expressed in murine lymphocytes. In addition, and as already mentioned by Affaticati et al., we did not detect NMDAR currents in T cells in patch clamp experiments (T. Bose, PhD thesis, 2014, (Kahlfuss et al., 2014)). Despite the lack of such functional proof, it cannot fully be excluded that NMADRs are expressed in lymphocytes at a very low level or only transiently and under specific conditions, for instance under cellular stress. NMDAR subunits in murine lymphocytes could exist in splice variants and post-translationally modified forms that are not detectable by the used Abs, although they detect NMDARs in neuronal cells. In the neuronal synapse the expression of NMDARs is low, often less than 10 molecules (Racca et al., 2000), and a single NMDAR pumps more Ca^{2+} than a single Stim-Orai complex of CRAC channels, the major Ca^{2+} channels in lymphocytes (Feske, 2007; Hogan et al., 2010). Thus, a very low expression of NMDARs in lymphocytes would make sense. However, I showed that the proliferation of thymocytes from GluN1 ko mice was inhibited by ifenprodil to a similar extent as proliferation of wt thymocytes. Hence, even if NMDARs are expressed and blocked by the antagonists, these should also target additional channels. K^+channels expressed on immune cells comprise Ca^{2+}-activated ($K_{Ca}3.1$), voltage-activated ($K_v1.3$) and two pore K_{2P} channels

(TASK1, TASK3), which mediate K^+-efflux and protect the membrane from depolarization. Ca^{2+}-activated $K_{Ca}3.1$ channels and two pore K^+channels have been shown to regulate immune cell effector function through hyperpolarizing the membrane, which favours Ca^{2+}-influx through CRAC channels (Cahalan and Chandy, 2009; Meuth et al., 2008). Other ion channels like transient receptor potential (TRP) family, voltage-gated Ca^{2+} (Cav) channels and P2X receptor channels also contribute to TCR-induced Ca^{2+}-flux in immune cells (Feske, 2013). In our collaborative project, we identified $K_v1.3$ and $K_{Ca}3.1$ channels as targets of the NMDAR antagonists ifenprodil, memantine and MK801 (T. Bose, PhD thesis, 2014, (Kahlfuss et al., 2014)). It is conceivable that cross-inhibition of these K^+channels results from their structural similarities with NMDARs and the resemblance of the $K_v1.3$ and $K_{Ca}3.1$ channel pore forming subunits with the binding sites of GluN1/N2A-C subunits of NMDARs. The competitive NMDAR antagonist D-APV, which inhibits neuronal NMDARs at 0.7 μM, had no effect on T-cell proliferation and showed no effect on the activity of the analysed K^+channels at concentrations up to 1 mM (Kahlfuss et al., 2014). These results further support the notion that NMDARs do not play a major role in murine lymphocytes, at least under the applied *in vitro* conditions.

4.2 The effects of NMDAR antagonists on T-cell signalling and proliferation

The NMDAR antagonists memantine and ketamine are used to treat advanced Alzheimer's disease and resistant depression, respectively (Olivares et al., 2012). Thus, besides their effects on neuronal activity, it is important to understand how NMDAR antagonists could impact on T- and B-cell responses. Using CD3 Ab-induced TCR triggering, I detected profound inhibitory effects of NMDAR antagonists on Th cell activation and proliferation. Addition of ifenprodil to short-term activated T cells (until 2 h) led to a significant attenuation of TCR-induced proximal, activation of the Src tyrosine kinases Lck/Fyn, and downstream signalling events including the activation of PLCγ1 and MAPK Erk1/2. In addition, activation of the critical Akt-mTOR-S6 signalling axis was also dampened. In long-term-stimulated CD4+ T cells ifenprodil treatment reduced the expression of pPLCγ1 and pGSK3β, implying an enhanced level of active GSK3β. In line with this and the reduced Ca^{2+}-flux in the presence of antagonists (Kahlfuss et al., 2014), the accumulation of nuclear NFATc1 was reduced by 65% upon ifenprodil treatment. pmTOR and pS6 levels were also downregulated, a further indication for an overall and long-lasting attenuation of TCR signalling in the presence of the drug. This reduction in TCR signalling strength correlated with a reduced T-

cell proliferation upon treatment with either ifenprodil, MK801 or memantine. The extent of inhibition depended on the strength of the TCR stimulus, i.e. the CD3 Ab concentration, and on the antagonist dose. When Th cells were activated with a weaker TCR stimulus (3 µg/ml Ab), the antagonists had a more profound effect and lower drug doses were sufficient to induce a significant inhibition of cell proliferation. Furthermore, ifenprodil's inhibitory effect on T-cell expansion was compensated by CD28 co-stimulation or exogenous IL-2, reflecting a suboptimal TCR-induced signalling and IL-2 production in the presence of the antagonist. CD28 signals strengthen TCR signals (Rudd et al., 2009; Weiss, 2009) and, accordingly, I found less reduction in pPLCγ1, pmTOR, and pS6 expression by ifenprodil when cells were co-stimulated with CD28 Abs. Thus, the extent of inhibitory effects of NMDAR antagonists on T-cell activation and proliferation is governed by the strength of the TCR stimulus, delivery of CD28 co-stimulation and the drug concentration. One could suppose that particularly naive T cells encountering low antigen doses or suboptimal co-stimulation by APCs or autoreactive T cells might be most sensitive to NMDAR antagonists.

In T and B cells $K_v1.3$ and $K_{Ca}3.1$ channels are involved in compensating the K^+-efflux needed for efficient Ca^{2+}-influx upon TCR or BCR ligation (Cahalan and Chandy, 2009; Conforti, 2012; Lam and Wulff, 2011). Inhibition of $K_v1.3$ and $K_{Ca}3.1$ channels by specific K^+ channel blockers inhibit T-cell function and modulate several immunological diseases, including rheumatoid arthritis (Toldi et al., 2013), experimental autoimmune encephalitis (EAE) (Li et al., 2012), and allergic inflammatory disease (Hua et al., 2013). $K_{Ca}3.1$ channels are functionally dominant in naive and early memory T cells, whereas $K_v1.3$ channels are the dominant K^+ channels in memory effector T cells. Interestingly, many of our results concerning the inhibition of T-cell activation and effector function by NMDAR antagonists (Kahlfuss et al., 2014) are resemble those reported for the inhibition of $K_v1.3$ channels (Hu et al., 2007; Hu et al., 2013; Wulff and Zhorov, 2008). This supports our conclusion that NMDAR antagonists like ifenprodil or memantine mainly function through the blockade of $K_v1.3$ and $K_{Ca}3.1$ channels. Since NMDAR antagonists cross-inhibit both types of K^+ channels, they may affect responses of naive as well as memory T cells. NMDAR antagonists may also modulate Treg function as Tregs express a similar number of $K_v1.3$ and $K_{Ca}3.1$ channels as naive T cells (Reneer et al., 2011; Varga et al., 2009). How NMDAR antagonists affect signalling processes in T effector and Treg cells is unknown and should be addressed in future studies.

Besides those K+ channels, NMDAR antagonists may also impair the function of other voltage-gated Ca^{2+}-channels, such as L-type channels or CRAC channels, through side effects on the steady state changes in membrane depolarisation. Indeed, MK801 has been shown to affect CRAC channel activity (Delaney et al., 2012; Feske, 2013; Omilusik et al., 2011; Shaw et al., 2013; Zainullina et al., 2011).

4.3 NMDAR antagonists in B-cell activation and function

We considered that, in addition to T cells, application of NMDAR antagonists could also cross-target B cells. A summary of the effects of NMDAR antgonists on T cells and B cells is given in Table I. We chose to activate B cells by ligation of the BCR through α-IgM Abs as well as LPS/TLR4 stimulation. The antagonists ifenprodil, memantine and MK801 impaired BCR- and LPS/TLR4-induced B-cell proliferation, but the competitive NMDAR antagonist D-APV had no significant influence on B-cell expansion. With respect to the drugs' influence on BCR-induced signalling, they attenuated the activation of Erk1/2, Akt, S6, and NFATc1, and thus seem to act in B cells via similar mechanisms as in T cells (Kahlfuss et al., 2014; Simma et al., 2014). In this context it is interesting that B cells stimulated with BCR plus CD40 Abs in the presence of ifenprodil still exhibited higher pErk1/2, pAkt and nuclear NFATc1 levels than B cells stimulated with α-IgM alone, although the drug induced a reduction in the activation of those molecules. This is consistent with the data from CD3 vs CD3+CD28 Ab-activated T cells. Despite attenuated signalling the overall signalling strength over time seems to be sufficient to allow proliferation of α-IgM/CD40 co-stimulated B cells in the presence of the antagonist.

Notably, the drugs also downregulated Erk1/2, Akt and NFATc1 activation of LPS/TLR4-stimulated B cells and blocked their proliferation. This may be of special relevance for innate immune responses of B cells, for instance in sepsis. Systemic inflammation induced by LPS also seems to affect neuronal pathology, for instance in MS and Parkinson’s disease (Cunningham, 2013; Danysz and Parsons, 2012; Martinez-Hernandez et al., 2011; Yanaba et al., 2008). Whether non-competitive NMDAR antagonists are suited to milden the course of these diseases is an interesting issue for future studies.

Besides proliferation, NMDAR antagonist ifenprodil inhibited the main function of B cells, Ab production. IgM as well as IgG secretion was almost totally aborted when the antagonist was added one day after LPS activation of B cells and it was still significantly reduced when ifenprodil was added at day 2. So far, we can only speculate that subtle changes in signalling

contribute to this effect and further experimentation is required to elucidate the exact mechanisms. However, it shows the potency of these drugs to also interfere with the responses of activated B cells. This may be therapeutically useful to block B cell hyperreactivity and autoantibody production in B cell-mediated chronic inflammation.

Function	Effect of NMDAR antagonist		
T cells:	ifenprodil	memantine	MK801
- proliferation	↓	↓	↓
- activation of Akt, Erk1/2, PLCγ1,S6, GSK3β, mTOR, NFATc1	↓		
- Ca^{2+}-flux	↓		
- $K_v1.3$ and $K_{Ca}3.1$ activity	↓	↓	↓
B cells:			
BCR-induced			
- proliferation	↓	↓	
- activation of Akt, Erk1/2, S6, NFATc1	↓		
- Ca^{2+}-flux	↓	↓	
- $K_v1.3$ and $K_{Ca}3.1$ activity	↓	↓	
BCR+CD40-induced			
- proliferation	↔		
- activation of Erk1/2, S6	↓		
- IL-10 production	↑		
LPS-induced:			
- proliferation	↓	↓	
- activation of Akt, Erk1/2, S6, NFATc1	↓		
- $K_v1.3$ and $K_{Ca}3.1$ activity	↓		
- IL-10 production	↔ ↓		
- IgM and IgD production (drug given at day 1)	↓		

Table 1. Summary of the effects of NMDAR antagonists on T- and B-cell signalling and function. Arrows indicate ↓ attenuation ↑ enhancement, ↔ no or little attenuation; green colour indicates data are provided by Dr. T. Bose (PhD thesis 2014; (Kahlfuss et al., 2014; Simma et al., 2014)).

Although B cells expressed mRNAs of NMDAR subunits, the detection of NMDARs on protein level by Western blot analysis was not feasible (data not shown), similar to what we found for T cells. As shown by my colleague Dr. T. Bose, the un- and non-competitive antagonists, but not D-APV, also blocked $K_v1.3$ and $K_{Ca}3.1$ channel activity of B cells and BCR-induced Ca^{2+}-flux (T. Bose, PhD thesis, 2014, (Kahlfuss et al., 2014)). Thus, the drugs act through cross-inhibition of those K^+ channels rather than specific inhibition of NMDARs

expressed on B cells, similar to the data from T cells. A reduced proliferation and class switch of memory B cells was found for specific K+ channel inhibitors (Matsumoto et al., 2011; Wulff et al., 2004). This supports our finding that the used un- and non-competitive NMDAR antagonists act on B cells via cross-inhibition of $K_v1.3$ and $K_{Ca}3.1$ channels.

Regulatory B10 cells control inflammation and immune responses by the temporary production of IL-10 (Maseda et al., 2012). B10 cells suppress Th1 differentiation and increase the number of $CD4^+CD25^+Foxp3^+$ regulatory T cells *in vivo* (Blair et al., 2009). Ifenprodil added to B cells 1 day after their activation with BCR/CD40 Abs fostered the generation of IL-10 producing B cells, whereas drug treatment at the begin of B cell stimulation (PMA/IO) abrogated IL-10 mRNA induction. How ifenprodil modulates B cell signalling in favour of IL-10 expression is curently unknown. The induction of IL-10 in B cells is complex and not fully understood. It is differentially controlled depending on the activation stimulus and availability of IL-21 (Candando et al., 2014). The deletion of ER Ca^{2+}-sensors STIM1 and STIM2 in B cells blocks IL-10 production resulting from a reduced Ca^{2+}-level after BCR ligation and a reduced induction of NFATp (NFAT1) (Matsumoto et al., 2011). Further molecules involved in the regulation of IL-10 induction are CREB (cAMP response element-binding protein), STAT3 and NFκB, the kinases Btk, CamKII, Erk1/2 and the adaptor protein SLP65 (Baba et al., 2014; Balagopalan et al., 2010; Jin et al., 2013). In B cells, the activation of Erk1/2 depends on PI-3K activation and Ca^{2+}-mobilization (Iritani et al., 1997; Jacob et al., 2002). In view that ifenprodil reduces BCR-induced Ca^{2+}-flux and NFATc1 activation and attenuates BCR/CD40-induced activation of Erk1/2 and Akt, we assume that addition of ifenprodil dampens signalling to a threshold favourable for IL-10 production.

Among the NMDAR antagonists used in this thesis only memantine is clinically applied in the treatment of Alzheimer's disease as it is well tolerated. In the mouse models of collagen induced arthritis and EAE, the mouse model for MS, memantine and MK801, respectively, mildened the disease (Bolton and Paul, 1997; Lindblad et al., 2012). Memantine also mitigated lung inflammatory responses and acute lung injury induced by bleomycin (Li et al., 2015). The mechanisms underlying disease amelioration, however, are unclear.

Here, I show that ifenprodil, MK801 and memantine are potent modulators of T- and B-lymphocyte proliferation, IgM/IgG secretion and B cell IL-10 production. The NMDAR antagonists act on lymphocytes through cross-inhibition of $K_v1.3$ and $K_{Ca}3.1$ channels and interfere with the activation of Akt-mTOR-S6, Erk1/2 and NFATc1 (Kahlfuss et al., 2014;

Simma et al., 2014), three critical arms of the T- and B-cell signalling network. The possibility of their oral application makes NMDAR antagonists, like memantine, attractive immunomodulatory or immunosuppressive drugs to control sytemic inflammation, autoimmune diseaes, B- and T-cell neoplasia or transplant rejection. Certainly, further *in vivo* studies in mouse models are needed for such translational approaches. In addition, the side effects of NMDAR antagonists on B- and T lymphocytes should be considered when they are used in treatments of neuronal disorders. Thus, further exciting findings on NMDAR antagonists influencing immune cell responses seem to lie ahead.

5. References

Ackermann, J.A., D. Radtke, A. Maurberger, T.H. Winkler, and L. Nitschke. 2011. Grb2 regulates B-cell maturation, B-cell memory responses and inhibits B-cell Ca2+ signalling. *The EMBO journal* 30:1621-1633.

Acuto, O., V. Di Bartolo, and F. Michel. 2008. Tailoring T-cell receptor signals by proximal negative feedback mechanisms. *Nature reviews. Immunology* 8:699-712.

Affaticati, P., O. Mignen, F. Jambou, M.C. Potier, I. Klingel-Schmitt, J. Degrouard, S. Peineau, E. Gouadon, G.L. Collingridge, R. Liblau, T. Capiod, and S. Cohen-Kaminsky. 2011. Sustained calcium signalling and caspase-3 activation involve NMDA receptors in thymocytes in contact with dendritic cells. *Cell death and differentiation* 18:99-108.

Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature immunology* 2:675-680.

Alexopoulou, L., A.C. Holt, R. Medzhitov, and R.A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732-738.

Amigorena, S., D. Choquet, J.L. Teillaud, H. Korn, and W.H. Fridman. 1990. Ion channel blockers inhibit B cell activation at a precise stage of the G1 phase of the cell cycle. Possible involvement of K+ channels. *J Immunol* 144:2038-2045.

Anderson, M., J.M. Suh, E.Y. Kim, and S.E. Dryer. 2011. Functional NMDA receptors with atypical properties are expressed in podocytes. *American journal of physiology. Cell physiology* 300:C22-32.

Aracava, Y., E.F. Pereira, A. Maelicke, and E.X. Albuquerque. 2005. Memantine blocks alpha7* nicotinic acetylcholine receptors more potently than n-methyl-D-aspartate receptors in rat hippocampal neurons. *The Journal of pharmacology and experimental therapeutics* 312:1195-1205.

Baba, Y., and T. Kurosaki. 2011. Impact of Ca2+ signaling on B cell function. *Trends in immunology* 32:589-594.

Baba, Y., M. Matsumoto, and T. Kurosaki. 2014. Calcium signaling in B cells: regulation of cytosolic Ca2+ increase and its sensor molecules, STIM1 and STIM2. *Molecular immunology* 62:339-343.

Baba, Y., M. Matsumoto, and T. Kurosaki. 2015. Signals controlling the development and activity of regulatory B-lineage cells. *International immunology*

Balagopalan, L., N.P. Coussens, E. Sherman, L.E. Samelson, and C.L. Sommers. 2010. The LAT story: a tale of cooperativity, coordination, and choreography. *Cold Spring Harbor perspectives in biology* 2:a005512.

Barann, M., H. Bonisch, B.W. Urban, and M. Gothert. 1998. Inhibition of 5-HT3 receptor cation channels by ifenprodil in excised patches of N1E-115 cells. *Naunyn-Schmiedeberg's archives of pharmacology* 358:145-152.

Bhattacharyya, S., J. Deb, A.K. Patra, D.A. Thuy Pham, W. Chen, M. Vaeth, F. Berberich-Siebelt, S. Klein-Hessling, E.D. Lamperti, K. Reifenberg, J. Jellusova, A. Schweizer, L. Nitschke, E. Leich, A. Rosenwald, C. Brunner, S. Engelmann, U. Bommhardt, A. Avots, M.R. Muller, E. Kondo, and E. Serfling. 2011. NFATc1 affects mouse splenic B cell function by controlling the calcineurin--NFAT signaling network. *The Journal of experimental medicine* 208:823-839.

Bittner, S., and S.G. Meuth. 2013. Targeting ion channels for the treatment of autoimmune neuroinflammation. *Therapeutic advances in neurological disorders* 6:322-336.

Blair, P.A., K.A. Chavez-Rueda, J.G. Evans, M.J. Shlomchik, A. Eddaoudi, D.A. Isenberg, M.R. Ehrenstein, and C. Mauri. 2009. Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice. *J Immunol* 182:3492-3502.

Boldyrev, A.A. 2005. Homocysteinic acid causes oxidative stress in lymphocytes by potentiating toxic effect of NMDA. *Bulletin of experimental biology and medicine* 140:33-37.

Boldyrev, A.A., D.O. Carpenter, and P. Johnson. 2005. Emerging evidence for a similar role of glutamate receptors in the nervous and immune systems. *Journal of neurochemistry* 95:913-918.

Boldyrev, A.A., V.I. Kazey, T.A. Leinsoo, A.P. Mashkina, O.V. Tyulina, P. Johnson, J.O. Tuneva, S. Chittur, and D.O. Carpenter. 2004. Rodent lymphocytes express functionally active glutamate receptors. *Biochemical and biophysical research communications* 324:133-139.

Bolton, C., and C. Paul. 1997. MK-801 limits neurovascular dysfunction during experimental allergic encephalomyelitis. *The Journal of pharmacology and experimental therapeutics* 282:397-402.

Bouaziz, J.D., K. Yanaba, and T.F. Tedder. 2008. Regulatory B cells as inhibitors of immune responses and inflammation. *Immunological reviews* 224:201-214.

Bozic, M., and J.M. Valdivielso. 2015. The potential of targeting NMDA receptors outside the CNS. *Expert opinion on therapeutic targets* 19:399-413.

Burkhardt, A.L., M. Brunswick, J.B. Bolen, and J.J. Mond. 1991. Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. *Proceedings of the National Academy of Sciences of the United States of America* 88:7410-7414.

Cahalan, M.D., and K.G. Chandy. 2009. The functional network of ion channels in T lymphocytes. *Immunological reviews* 231:59-87.

Candando, K.M., J.M. Lykken, and T.F. Tedder. 2014. B10 cell regulation of health and disease. *Immunological reviews* 259:259-272.

Chakraborty, A.K., and A. Weiss. 2014. Insights into the initiation of TCR signaling. *Nature immunology* 15:798-807.

Chaplin, D.D. 2006. 1. Overview of the human immune response. *The Journal of allergy and clinical immunology* 117:S430-435.

Chenu, C., C.M. Serre, C. Raynal, B. Burt-Pichat, and P.D. Delmas. 1998. Glutamate receptors are expressed by bone cells and are involved in bone resorption. *Bone* 22:295-299.

Chuvpilo, S., E. Jankevics, D. Tyrsin, A. Akimzhanov, D. Moroz, M.K. Jha, J. Schulze-Luehrmann, B. Santner-Nanan, E. Feoktistova, T. Konig, A. Avots, E. Schmitt, F. Berberich-Siebelt, A. Schimpl, and E. Serfling. 2002. Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. *Immunity* 16:881-895.

Conforti, L. 2012. The ion channel network in T lymphocytes, a target for immunotherapy. *Clin Immunol* 142:105-106.

Conn, P.J. 2003. Physiological roles and therapeutic potential of metabotropic glutamate receptors. *Annals of the New York Academy of Sciences* 1003:12-21.

Cross, D.A., D.R. Alessi, P. Cohen, M. Andjelkovich, and B.A. Hemmings. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785-789.

Cunningham, C. 2013. Microglia and neurodegeneration: the role of systemic inflammation. *Glia* 61:71-90.

Daggett, A., and X.W. Yang. 2013. Huntington's disease: easing the NMDAR traffic jam. *Nature medicine* 19:971-973.

Danysz, W., and C.G. Parsons. 2012. Alzheimer's disease, beta-amyloid, glutamate, NMDA receptors and memantine--searching for the connections. *British journal of pharmacology* 167:324-352.

Delaney, A.J., J.M. Power, and P. Sah. 2012. Ifenprodil reduces excitatory synaptic transmission by blocking presynaptic P/Q type calcium channels. *Journal of neurophysiology* 107:1571-1575.

Diehn, M., A.A. Alizadeh, O.J. Rando, C.L. Liu, K. Stankunas, D. Botstein, G.R. Crabtree, and P.O. Brown. 2002. Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. In Proceedings of the National Academy of Sciences of the United States of America. 11796-11801.

Engel, I., and C. Murre. 2001. The function of E- and Id proteins in lymphocyte development. *Nature reviews. Immunology* 1:193-199.

Ferraguti, F., and R. Shigemoto. 2006. Metabotropic glutamate receptors. *Cell and tissue research* 326:483-504.

Feske, S. 2007. Calcium signalling in lymphocyte activation and disease. *Nature reviews. Immunology* 7:690-702.

Feske, S. 2013. Ca(2+) influx in T cells: how many ca(2+) channels? *Frontiers in immunology* 4:99.

Fillatreau, S., C.H. Sweenie, M.J. McGeachy, D. Gray, and S.M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nature immunology* 3:944-950.

Finke, C., U.A. Kopp, M. Scheel, L.M. Pech, C. Soemmer, J. Schlichting, F. Leypoldt, A.U. Brandt, J. Wuerfel, C. Probst, C.J. Ploner, H. Pruss, and F. Paul. 2013. Functional and structural brain changes in anti-N-methyl-D-aspartate receptor encephalitis. *Annals of neurology* 74:284-296.

Fuller, D.M., M. Zhu, C.W. Ou-Yang, S.A. Sullivan, and W. Zhang. 2011. A tale of two TRAPs: LAT and LAB in the regulation of lymphocyte development, activation, and autoimmunity. *Immunologic research* 49:97-108.

Ganor, Y., M. Besser, N. Ben-Zakay, T. Unger, and M. Levite. 2003. Human T cells express a functional ionotropic glutamate receptor GluR3, and glutamate by itself triggers integrin-mediated adhesion to laminin and fibronectin and chemotactic migration. *J Immunol* 170:4362-4372.

Ganor, Y., and M. Levite. 2014. The neurotransmitter glutamate and human T cells: glutamate receptors and glutamate-induced direct and potent effects on normal human T cells, cancerous human leukemia and lymphoma T cells, and autoimmune human T cells. *J Neural Transm* 121:983-1006.

Genever, P.G., D.J. Wilkinson, A.J. Patton, N.M. Peet, Y. Hong, A. Mathur, J.D. Erusalimsky, and T.M. Skerry. 1999. Expression of a functional N-methyl-D-aspartate-type glutamate receptor by bone marrow megakaryocytes. *Blood* 93:2876-2883.

Germain, R.N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nature reviews. Immunology* 2:309-322.

Groc, L., M. Heine, S.L. Cousins, F.A. Stephenson, B. Lounis, L. Cognet, and D. Choquet. 2006. NMDA receptor surface mobility depends on NR2A-2B subunits. *Proceedings of the National Academy of Sciences of the United States of America* 103:18769-18774.

Gwack, Y., S. Feske, S. Srikanth, P.G. Hogan, and A. Rao. 2007. Signalling to transcription: store-operated Ca2+ entry and NFAT activation in lymphocytes. *Cell calcium* 42:145-156.

Harnett, M.M. 2004. CD40: a growing cytoplasmic tale. *Science's STKE : signal transduction knowledge environment* 2004:pe25.

Hayashi, F., K.D. Smith, A. Ozinsky, T.R. Hawn, E.C. Yi, D.R. Goodlett, J.K. Eng, S. Akira, D.M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410:1099-1103.

Hock, M., M. Vaeth, R. Rudolf, A.K. Patra, D.A. Pham, K. Muhammad, T. Pusch, T. Bopp, E. Schmitt, R. Rost, F. Berberich-Siebelt, D. Tyrsin, S. Chuvpilo, A. Avots, E. Serfling, and S. Klein-Hessling. 2013. NFATc1 induction in peripheral T and B lymphocytes. *J Immunol* 190:2345-2353.

Hogan, P.G., R.S. Lewis, and A. Rao. 2010. Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. *Annual review of immunology* 28:491-533.

Hogan, P.G., and A. Rao. 2015. Store-operated calcium entry: Mechanisms and modulation. *Biochemical and biophysical research communications* 460:40-49.

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 162:3749-3752.

Hu, L., M. Pennington, Q. Jiang, K.A. Whartenby, and P.A. Calabresi. 2007. Characterization of the functional properties of the voltage-gated potassium channel Kv1.3 in human CD4+ T lymphocytes. *J Immunol* 179:4563-4570.

Hu, L., T. Wang, A.R. Gocke, A. Nath, H. Zhang, J.B. Margolick, K.A. Whartenby, and P.A. Calabresi. 2013. Blockade of Kv1.3 potassium channels inhibits differentiation and granzyme B secretion of human CD8+ T effector memory lymphocytes. *PloS one* 8:e54267.

Hua, X., T. Deuse, Y.J. Chen, H. Wulff, M. Stubbendorff, R. Kohler, H. Miura, F. Langer, H. Reichenspurner, R.C. Robbins, and S. Schrepfer. 2013. The potassium channel KCa3.1 as new therapeutic target for the prevention of obliterative airway disease. *Transplantation* 95:285-292.

Inagaki, N., H. Kuromi, T. Gonoi, Y. Okamoto, H. Ishida, Y. Seino, T. Kaneko, T. Iwanaga, and S. Seino. 1995. Expression and role of ionotropic glutamate receptors in pancreatic islet cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 9:686-691.

Iravani, M.M., R. Muscat, and Z.L. Kruk. 1999. MK-801 interaction with the 5-HT transporter: a real-time study in brain slices using fast cyclic voltammetry. *Synapse* 32:212-224.

Iritani, B.M., K.A. Forbush, M.A. Farrar, and R.M. Perlmutter. 1997. Control of B cell development by Ras-mediated activation of Raf. *The EMBO journal* 16:7019-7031.

Isakov, N., R.L. Wange, W.H. Burgess, J.D. Watts, R. Aebersold, and L.E. Samelson. 1995. ZAP-70 binding specificity to T cell receptor tyrosine-based activation motifs: the tandem SH2 domains of ZAP-70 bind distinct tyrosine-based activation motifs with varying affinity. *The Journal of experimental medicine* 181:375-380.

Jacob, A., D. Cooney, M. Pradhan, and K.M. Coggeshall. 2002. Convergence of signaling pathways on the activation of ERK in B cells. *The Journal of biological chemistry* 277:23420-23426.

Janeway, C.A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annual review of immunology* 20:197-216.

Jin, G., Y. Hamaguchi, T. Matsushita, M. Hasegawa, D. Le Huu, N. Ishiura, K. Naka, A. Hirao, K. Takehara, and M. Fujimoto. 2013. B-cell linker protein expression contributes to controlling allergic and autoimmune diseases by mediating IL-10 production in regulatory B cells. *The Journal of allergy and clinical immunology* 131:1674-1682.

Johnson, J.W., and P. Ascher. 1987. Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 325:529-531.

Kahlfuss, S. 2015. Der Einfluss von NMDA-Rezeptor-Antagonisten auf durch dendritische Zellen induzierte CD4+ T-Zellfunctionen. In PhD thesis, Magdeburg, Germany.

Kahlfuss, S., N. Simma, J. Mankiewicz, T. Bose, T. Lowinus, S. Klein-Hessling, R. Sprengel, B. Schraven, M. Heine, and U. Bommhardt. 2014. Immunosuppression by N-methyl-D-aspartate receptor antagonists is mediated through inhibition of Kv1.3 and KCa3.1 channels in T cells. *Molecular and cellular biology* 34:820-831.

Kamanaka, M., S.T. Kim, Y.Y. Wan, F.S. Sutterwala, M. Lara-Tejero, J.E. Galan, E. Harhaj, and R.A. Flavell. 2006. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. *Immunity* 25:941-952.

Kawai, T., and S. Akira. 2006. TLR signaling. *Cell death and differentiation* 13:816-825.

Klein, L., B. Kyewski, P.M. Allen, and K.A. Hogquist. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature reviews. Immunology* 14:377-391.

Kleopa, K.A. 2011. Autoimmune channelopathies of the nervous system. *Current neuropharmacology* 9:458-467.

Kliche, S., D. Breitling, M. Togni, R. Pusch, K. Heuer, X. Wang, C. Freund, A. Kasirer-Friede, G. Menasche, G.A. Koretzky, and B. Schraven. 2006. The ADAP/SKAP55 signaling module regulates T-cell receptor-mediated integrin activation through plasma membrane targeting of Rap1. *Molecular and cellular biology* 26:7130-7144.

Kobayashi, T., K. Washiyama, and K. Ikeda. 2006. Inhibition of G protein-activated inwardly rectifying K+ channels by ifenprodil. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 31:516-524.

Kraus, M., M.B. Alimzhanov, N. Rajewsky, and K. Rajewsky. 2004. Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer. *Cell* 117:787-800.

Kurosaki, T. 1999. Genetic analysis of B cell antigen receptor signaling. *Annual review of immunology* 17:555-592.

Lam, J., and H. Wulff. 2011. The Lymphocyte Potassium Channels Kv1.3 and KCa3.1 as Targets for Immunosuppression. *Drug development research* 72:573-584.

Lee, M.S., and Y.J. Kim. 2007. Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annual review of biochemistry* 76:447-480.

Lennon, V.A., T.J. Kryzer, G.E. Griesmann, P.E. O'Suilleabhain, A.J. Windebank, A. Woppmann, G.P. Miljanich, and E.H. Lambert. 1995. Calcium-channel antibodies in the Lambert-Eaton syndrome and other paraneoplastic syndromes. *The New England journal of medicine* 332:1467-1474.

Levite, M. 2008. Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. *Current opinion in pharmacology* 8:460-471.

Lewis, R.S., and M.D. Cahalan. 1995. Potassium and calcium channels in lymphocytes. *Annual review of immunology* 13:623-653.

Li, Y., Y. Liu, X. Peng, W. Liu, F. Zhao, D. Feng, J. Han, Y. Huang, S. Luo, L. Li, S.J. Yue, Q. Cheng, X. Huang, and Z. Luo. 2015. NMDA Receptor Antagonist Attenuates Bleomycin-Induced Acute Lung Injury. *PloS one* 10:e0125873.

Li, Z., L. Chen, X. Niu, J. Liu, M. Ping, R. Li, X. Xie, and L. Guo. 2012. Immunomodulatory synergy by combining atorvastatin and rapamycin in the treatment of experimental autoimmune encephalomyelitis (EAE). *Journal of neuroimmunology* 250:9-17.

Lindblad, S.S., P. Mydel, A. Hellvard, I.M. Jonsson, and M.I. Bokarewa. 2012. The N-methyl-d-aspartic acid receptor antagonist memantine ameliorates and delays the development of arthritis by enhancing regulatory T cells. *Neuro-Signals* 20:61-71.

Lipton, S.A. 2004. Paradigm shift in NMDA receptor antagonist drug development: molecular mechanism of uncompetitive inhibition by memantine in the treatment of Alzheimer's disease and other neurologic disorders. *Journal of Alzheimer's disease : JAD* 6:S61-74.

Liu, S.K., N. Fang, G.A. Koretzky, and C.J. McGlade. 1999. The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. *Current biology : CB* 9:67-75.

Lombardi, G., C. Dianzani, G. Miglio, P.L. Canonico, and R. Fantozzi. 2001. Characterization of ionotropic glutamate receptors in human lymphocytes. *British journal of pharmacology* 133:936-944.

Love, P.E., and S.M. Hayes. 2010. ITAM-mediated signaling by the T-cell antigen receptor. *Cold Spring Harbor perspectives in biology* 2:a002485.

Lueg, G., C.C. Gross, H. Lohmann, A. Johnen, A. Kemmling, M. Deppe, J. Groger, J. Minnerup, H. Wiendl, S.G. Meuth, and T. Duning. 2015. Clinical relevance of specific T-cell activation in the blood and cerebrospinal fluid of patients with mild Alzheimer's disease. *Neurobiology of aging* 36:81-89.

Machado-Vieira, R., G. Salvadore, N. DiazGranados, L. Ibrahim, D. Latov, C. Wheeler-Castillo, J. Baumann, I.D. Henter, and C.A. Zarate, Jr. 2010. New therapeutic targets for mood disorders. *TheScientificWorldJournal* 10:713-726.

Malinow, R. 2012. New developments on the role of NMDA receptors in Alzheimer's disease. *Current opinion in neurobiology* 22:559-563.

Martinez-Hernandez, E., J. Horvath, Y. Shiloh-Malawsky, N. Sangha, M. Martinez-Lage, and J. Dalmau. 2011. Analysis of complement and plasma cells in the brain of patients with anti-NMDAR encephalitis. *Neurology* 77:589-593.

Maseda, D., S.H. Smith, D.J. DiLillo, J.M. Bryant, K.M. Candando, C.T. Weaver, and T.F. Tedder. 2012. Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo. *J Immunol* 188:1036-1048.

Matsumoto, M., Y. Fujii, A. Baba, M. Hikida, T. Kurosaki, and Y. Baba. 2011. The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production. *Immunity* 34:703-714.

Matsushita, T., K. Yanaba, J.D. Bouaziz, M. Fujimoto, and T.F. Tedder. 2008. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *The Journal of clinical investigation* 118:3420-3430.

Mauri, C., D. Gray, N. Mushtaq, and M. Londei. 2003. Prevention of arthritis by interleukin 10-producing B cells. *The Journal of experimental medicine* 197:489-501.

Mayer, M.L. 2005. Glutamate receptor ion channels. *Current opinion in neurobiology* 15:282-288.

Mayer, M.L., G.L. Westbrook, and P.B. Guthrie. 1984. Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature* 309:261-263.

Melchers, F. 2005. The pre-B-cell receptor: selector of fitting immunoglobulin heavy chains for the B-cell repertoire. *Nature reviews. Immunology* 5:578-584.

Meuth, S.G., S. Bittner, P. Meuth, O.J. Simon, T. Budde, and H. Wiendl. 2008. TWIK-related acid-sensitive K+ channel 1 (TASK1) and TASK3 critically influence T lymphocyte effector functions. *The Journal of biological chemistry* 283:14559-14570.

Miglio, G., C. Dianzani, S. Fallarini, R. Fantozzi, and G. Lombardi. 2007. Stimulation of N-methyl-D-aspartate receptors modulates Jurkat T cell growth and adhesion to fibronectin. *Biochemical and biophysical research communications* 361:404-409.

Miglio, G., F. Varsaldi, and G. Lombardi. 2005. Human T lymphocytes express N-methyl-D-aspartate receptors functionally active in controlling T cell activation. *Biochemical and biophysical research communications* 338:1875-1883.

Murphy, K., Janeway Jr, CA., Travers, P., Walport, M. 2012. Janeway Immunobiology. Garland Science, New York.

Nakanishi, S. 1992. Molecular diversity of glutamate receptors and implications for brain function. *Science* 258:597-603.

Nakayama, T., and M. Yamashita. 2010. The TCR-mediated signaling pathways that control the direction of helper T cell differentiation. *Seminars in immunology* 22:303-309.

Niswender, C.M., and P.J. Conn. 2010. Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annual review of pharmacology and toxicology* 50:295-322.

Okkenhaug, K., M. Turner, and M.R. Gold. 2014. PI3K Signaling in B Cell and T Cell Biology. *Frontiers in immunology* 5:557.

Olivares, D., V.K. Deshpande, Y. Shi, D.K. Lahiri, N.H. Greig, J.T. Rogers, and X. Huang. 2012. N-methyl D-aspartate (NMDA) receptor antagonists and memantine treatment for Alzheimer's disease, vascular dementia and Parkinson's disease. *Current Alzheimer research* 9:746-758.

Omilusik, K., J.J. Priatel, X. Chen, Y.T. Wang, H. Xu, K.B. Choi, R. Gopaul, A. McIntyre-Smith, H.S. Teh, R. Tan, N.T. Bech-Hansen, D. Waterfield, D. Fedida, S.V. Hunt, and W.A. Jefferies. 2011. The Ca(v)1.4 calcium channel is a critical regulator of T cell receptor signaling and naive T cell homeostasis. *Immunity* 35:349-360.

Orser, B.A., P.S. Pennefather, and J.F. MacDonald. 1997. Multiple mechanisms of ketamine blockade of N-methyl-D-aspartate receptors. *Anesthesiology* 86:903-917.

Pacheco, R., T. Gallart, C. Lluis, and R. Franco. 2007. Role of glutamate on T-cell mediated immunity. *Journal of neuroimmunology* 185:9-19.

Pacheco, R., H. Oliva, J.M. Martinez-Navio, N. Climent, F. Ciruela, J.M. Gatell, T. Gallart, J. Mallol, C. Lluis, and R. Franco. 2006. Glutamate released by dendritic cells as a novel modulator of T cell activation. *J Immunol* 177:6695-6704.

Paoletti, P., C. Bellone, and Q. Zhou. 2013. NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nature reviews. Neuroscience* 14:383-400.

Paoletti, P., and J. Neyton. 2007. NMDA receptor subunits: function and pharmacology. *Current opinion in pharmacology* 7:39-47.

Partiseti, M., D. Choquet, A. Diu, and H. Korn. 1992. Differential regulation of voltage- and calcium-activated potassium channels in human B lymphocytes. *J Immunol* 148:3361-3368.

Patra, A.K., A. Avots, R.P. Zahedi, T. Schuler, A. Sickmann, U. Bommhardt, and E. Serfling. 2013. An alternative NFAT-activation pathway mediated by IL-7 is critical for early thymocyte development. *Nature immunology* 14:127-135.

Patra, A.K., T. Drewes, S. Engelmann, S. Chuvpilo, H. Kishi, T. Hunig, E. Serfling, and U.H. Bommhardt. 2006. PKB rescues calcineurin/NFAT-induced arrest of Rag expression and pre-T cell differentiation. *J Immunol* 177:4567-4576.

Paz, P.E., S. Wang, H. Clarke, X. Lu, D. Stokoe, and A. Abo. 2001. Mapping the Zap-70 phosphorylation sites on LAT (linker for activation of T cells) required for recruitment and activation of signalling proteins in T cells. *The Biochemical journal* 356:461-471.

Pierau, M., S.Y. Na, N. Simma, T. Lowinus, A. Marx, B. Schraven, and U.H. Bommhardt. 2012. Constitutive Akt1 signals attenuate B-cell receptor signaling and proliferation, but enhance B-cell migration and effector function. *European journal of immunology* 42:3381-3393.

Poe, J.C., S.H. Smith, K.M. Haas, K. Yanaba, T. Tsubata, T. Matsushita, and T.F. Tedder. 2011. Amplified B lymphocyte CD40 signaling drives regulatory B10 cell expansion in mice. *PloS one* 6:e22464.

Racca, C., F.A. Stephenson, P. Streit, J.D. Roberts, and P. Somogyi. 2000. NMDA receptor content of synapses in stratum radiatum of the hippocampal CA1 area. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20:2512-2522.

Ramanathan, S., S.S. Mohammad, F. Brilot, and R.C. Dale. 2014. Autoimmune encephalitis: recent updates and emerging challenges. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia* 21:722-730.

Rammes, G., R. Rupprecht, U. Ferrari, W. Zieglgansberger, and C.G. Parsons. 2001. The N-methyl-D-aspartate receptor channel blockers memantine, MRZ 2/579 and other amino-alkyl-cyclohexanes antagonise 5-HT(3) receptor currents in cultured HEK-293 and N1E-115 cell systems in a non-competitive manner. *Neuroscience letters* 306:81-84.

Rao, A., C. Luo, and P.G. Hogan. 1997. Transcription factors of the NFAT family: regulation and function. *Annual review of immunology* 15:707-747.

Rauch, P.J., A. Chudnovskiy, C.S. Robbins, G.F. Weber, M. Etzrodt, I. Hilgendorf, E. Tiglao, J.L. Figueiredo, Y. Iwamoto, I. Theurl, R. Gorbatov, M.T. Waring, A.T. Chicoine, M. Mouded, M.J. Pittet, M. Nahrendorf, R. Weissleder, and F.K. Swirski. 2012. Innate response activator B cells protect against microbial sepsis. *Science* 335:597-601.

Reneer, M.C., D.J. Estes, A.C. Velez-Ortega, A. Norris, M. Mayer, and F. Marti. 2011. Peripherally induced human regulatory T cells uncouple Kv1.3 activation from TCR-associated signaling. *European journal of immunology* 41:3170-3175.

Robert, V., E. Triffaux, M. Savignac, and L. Pelletier. 2011. Calcium signalling in T-lymphocytes. *Biochimie* 93:2087-2094.

Rogers, S.W., P.I. Andrews, L.C. Gahring, T. Whisenand, K. Cauley, B. Crain, T.E. Hughes, S.F. Heinemann, and J.O. McNamara. 1994. Autoantibodies to glutamate receptor GluR3 in Rasmussen's encephalitis. *Science* 265:648-651.

Rolli, V., M. Gallwitz, T. Wossning, A. Flemming, W.W. Schamel, C. Zurn, and M. Reth. 2002. Amplification of B cell antigen receptor signaling by a Syk/ITAM positive feedback loop. *Molecular cell* 10:1057-1069.

Rudd, C.E., A. Taylor, and H. Schneider. 2009. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunological reviews* 229:12-26.

Rudolf, R., R. Busch, A.K. Patra, K. Muhammad, A. Avots, J.C. Andrau, S. Klein-Hessling, and E. Serfling. 2014. Architecture and expression of the nfatc1 gene in lymphocytes. *Frontiers in immunology* 5:21.

Salter, M.W., Y. Dong, L.V. Kalia, X.J. Liu, and G. Pitcher. 2009. Regulation of NMDA Receptors by Kinases and Phosphatases. In Biology of the NMDA Receptor. A.M. Van Dongen, editor Boca Raton (FL).

Sansing, L.H., E. Tuzun, M.W. Ko, J. Baccon, D.R. Lynch, and J. Dalmau. 2007. A patient with encephalitis associated with NMDA receptor antibodies. *Nature clinical practice. Neurology* 3:291-296.

Sarchielli, P., M. Di Filippo, A. Candeliere, D. Chiasserini, A. Mattioni, S. Tenaglia, M. Bonucci, and P. Calabresi. 2007. Expression of ionotropic glutamate receptor GLUR3 and effects of glutamate on MBP- and MOG-specific lymphocyte activation and chemotactic migration in multiple sclerosis patients. *Journal of neuroimmunology* 188:146-158.

Schwenk, F., U. Baron, and K. Rajewsky. 1995. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic acids research* 23:5080-5081.

Seeman, P., C. Caruso, and M. Lasaga. 2008. Dopamine partial agonist actions of the glutamate receptor agonists LY 354,740 and LY 379,268. *Synapse* 62:154-158.

Serfling, E., F. Berberich-Siebelt, and A. Avots. 2007. NFAT in lymphocytes: a factor for all events? *Science's STKE : signal transduction knowledge environment* 2007:pe42.

Serfling, E., S. Klein-Hessling, A. Palmetshofer, T. Bopp, M. Stassen, and E. Schmitt. 2006. NFAT transcription factors in control of peripheral T cell tolerance. *European journal of immunology* 36:2837-2843.

Sharma, S., G.M. Findlay, H.S. Bandukwala, S. Oberdoerffer, B. Baust, Z. Li, V. Schmidt, P.G. Hogan, D.B. Sacks, and A. Rao. 2011. Dephosphorylation of the nuclear factor of activated T cells (NFAT) transcription factor is regulated by an RNA-protein scaffold complex. *Proceedings of the National Academy of Sciences of the United States of America* 108:11381-11386.

Shaw, P.J., B. Qu, M. Hoth, and S. Feske. 2013. Molecular regulation of CRAC channels and their role in lymphocyte function. *Cellular and molecular life sciences : CMLS* 70:2637-2656.

Shleper, M., E. Kartvelishvily, and H. Wolosker. 2005. D-serine is the dominant endogenous coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25:9413-9417.

Shohami, E., and A. Biegon. 2014. Novel approach to the role of NMDA receptors in traumatic brain injury. *CNS & neurological disorders drug targets* 13:567-573.

Simma, N., T. Bose, S. Kahlfuss, J. Mankiewicz, T. Lowinus, F. Luhder, T. Schuler, B. Schraven, M. Heine, and U. Bommhardt. 2014. NMDA-receptor antagonists block B-cell function but foster IL-10 production in BCR/CD40-activated B cells. *Cell communication and signaling : CCS* 12:75.

Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan. 2009. T cell activation. *Annual review of immunology* 27:591-619.

Sprengel, R., and F.N. Single. 1999. Mice with genetically modified NMDA and AMPA receptors. *Annals of the New York Academy of Sciences* 868:494-501.

Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. *Annual review of immunology* 21:139-176.

Swat, W., L. Ignatowicz, H. von Boehmer, and P. Kisielow. 1991. Clonal deletion of immature CD4+8+ thymocytes in suspension culture by extrathymic antigen-presenting cells. *Nature* 351:150-153.

Sylvester, M., S. Kliche, S. Lange, S. Geithner, C. Klemm, A. Schlosser, A. Grossmann, U. Stelzl, B. Schraven, E. Krause, and C. Freund. 2010. Adhesion and degranulation promoting adapter protein (ADAP) is a central hub for phosphotyrosine-mediated interactions in T cells. *PloS one* 5:e11708.

T. Bose. 2014. Crosstalk between NMDAR Antagonists and Potassium Channels in Murine and Human Lymphocytes. In PhD thesis, Magdeburg, Germany.

ten Boekel, E., F. Melchers, and A.G. Rolink. 1998. Precursor B cells showing H chain allelic inclusion display allelic exclusion at the level of pre-B cell receptor surface expression. *Immunity* 8:199-207.

Toldi, G., A. Bajnok, D. Dobi, A. Kaposi, L. Kovacs, B. Vasarhelyi, and A. Balog. 2013. The effects of Kv1.3 and IKCa1 potassium channel inhibition on calcium influx of human peripheral T lymphocytes in rheumatoid arthritis. *Immunobiology* 218:311-316.

Tomek, S.E., A.L. Lacrosse, N.E. Nemirovsky, and M.F. Olive. 2013. NMDA Receptor Modulators in the Treatment of Drug Addiction. *Pharmaceuticals (Basel)* 6:251-268.

van Zelm, M.C., T. Szczepanski, M. van der Burg, and J.J. van Dongen. 2007. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. *The Journal of experimental medicine* 204:645-655.

Varga, Z., T. Csepany, F. Papp, A. Fabian, P. Gogolak, A. Toth, and G. Panyi. 2009. Potassium channel expression in human CD4+ regulatory and naive T cells from healthy subjects and multiple sclerosis patients. *Immunology letters* 124:95-101.

Wang, Y., S.R. Brooks, X. Li, A.N. Anzelon, R.C. Rickert, and R.H. Carter. 2002. The physiologic role of CD19 cytoplasmic tyrosines. *Immunity* 17:501-514.

Weiss, A. 2009. TCR signal transduction: opening the black box. *J Immunol* 183:4821-4827.

Wienands, J., and N. Engels. 2001. Multitasking of Ig-alpha and Ig-beta to regulate B cell antigen receptor function. *International reviews of immunology* 20:679-696.

Wisden, W., and P.H. Seeburg. 1993. Mammalian ionotropic glutamate receptors. *Current opinion in neurobiology* 3:291-298.

Wulff, H., H.G. Knaus, M. Pennington, and K.G. Chandy. 2004. K+ channel expression during B cell differentiation: implications for immunomodulation and autoimmunity. *J Immunol* 173:776-786.

Wulff, H., and B.S. Zhorov. 2008. K+ channel modulators for the treatment of neurological disorders and autoimmune diseases. *Chemical reviews* 108:1744-1773.

Yan, Q., T. Barros, P.R. Visperas, S. Deindl, T.A. Kadlecek, A. Weiss, and J. Kuriyan. 2013. Structural basis for activation of ZAP-70 by phosphorylation of the SH2-kinase linker. *Molecular and cellular biology* 33:2188-2201.

Yanaba, K., J.D. Bouaziz, T. Matsushita, C.M. Magro, E.W. St Clair, and T.F. Tedder. 2008. B-lymphocyte contributions to human autoimmune disease. *Immunological reviews* 223:284-299.

Yanaba, K., J.D. Bouaziz, T. Matsushita, T. Tsubata, and T.F. Tedder. 2009. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. *J Immunol* 182:7459-7472.

Yoshizaki, A., T. Miyagaki, D.J. DiLillo, T. Matsushita, M. Horikawa, E.I. Kountikov, R. Spolski, J.C. Poe, W.J. Leonard, and T.F. Tedder. 2012. Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature* 491:264-268.

Yuan, H., C.M. Low, O.A. Moody, A. Jenkins, and S.F. Traynelis. 2015. Ionotropic GABA and Glutamate Receptor Mutations and Human Neurological Diseases. *Molecular pharmacology*

Zainullina, L.F., R.S. Yamidanov, V.A. Vakhitov, and Y.V. Vakhitova. 2011. NMDA receptors as a possible component of store-operated Ca(2)(+) entry in human T-lymphocytes. *Biochemistry. Biokhimiia* 76:1220-1226.

Zhu, J., and W.E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112:1557-1569.

6. Abbreviations

Abs	antibodies
Akt	Ak thymoma kinase
APC	antigen presenting cell
BCR	B cell receptor
CaM	calmodulin
CaMK	calmodulin kinase
CD	cluster of differentiation
CE	cytoplasmic protein extract
CLP	common lymphoid progenitor
CNS	central nervous system
CRAC	calcium release activated channel
CTL	cytotoxic T lymphocyte
DAG	diacylglycerol
DC	dendritic cell
DN	double negative
DP	double positive
ER	endoplasmic reticulum
Erk1/2	extracellular signal-regulated kinase 1/2
FACS	fluorescence activated cell sorting
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glu	glutamate
GluR	glutamate receptor
GSK3β	glycogen synthetase kinase 3 β
IFN-γ	interferon gamma
Ig	immunoglobulin
iGluR	ionotropic glutamate receptor
IO	ionomycin
IL	interleukin
IP3	inositol 1, 4, 5-triphosphate
ko	knockout
Lck	lymphocyte-specific protein tyrosine kinase

LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MHC	major histocompatibility complex
mGluR	metabotropic glutamate receptor
mTOR	mammalian target of rapamycin
MW	molecular weight
NE	nucelar protein extract
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa light chain enhancer of activated B cells
NMDAR	N-methyl-D-aspartate receptor
PBS	phosphate buffered saline
PI3-K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol-4, 5-biphosphate
PIP3	phosphatidylinositol-3, 4, 5-triphosphate
PKC	protein kinase C
PLCγ	phospholipase C gamma
PMA	phorbol-12-myistate-acetate
rpm	revolution per minute
RT	room temperature
SP	single positive
Src	sarcoma tyrosine kinase
STIM1/2	sensor stromal interaction molecule 1/2
Syk	spleen tyrosine kinase
TCR	T cell receptor
tg	transgenic
Th cell	T helper cell
TLR4	Toll like receptor 4
Treg	regulatory T cell
wt	wildtype

7. List of Figures

8. Erklärung

M.Sc. / Simma, Narasimhulu
Tel.. +49-(0)-17683350265
Address: Institute for Molecular and Clinical Immunology (IMKI),
Leipziger Str. 44, 39120 Magdeburg
E-mail: narasimhulu.simma@st.ovgu.de

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zum dem Thema

"The Role of NMDA-Receptors (NMDARs) and NMDAR Antagonists in Murine T- and B-Lymphocyte Function"

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades Dr. rer. nat. an anderen Einrichtungen eingereicht habe.

Mageburg, 23.06.2015 Narasimhulu Simma

9. Curriculum Vitae

Name & surname: Narasimhulu Simma

Date of birth: 16-08-1981

Place of birth: Srikakulam, India

Gender: male

Family status: married, 1 child

Nationality: Indian

Current address: Fermersleber Weg 45e, Zi. 501, 39112 Magdeburg

E-mail: narasimhulu.simma@st.ovgu.de

Education:

12/2010 -12/2014	PhD work in Immunology at the Institute for Molecular and Clinical Immunology, Medical Faculty of the Otto-von-Guericke-University, Magdeburg, Germany; Research Program within the SFB854 "Molecular Organization of Cellular Communication in the Immune System"
01/2008-05/2010	Undergraduate studies in Biology with specialization in Molecular Biology; Master degree at University of Skovde, Skovde, Sweden
07/2001-05/2003	Undergraduate studies in Biology with specialization in Biochemistry; Master degree at Andhra University, Visakhapatnam, India
06/1998-05/2001	Undergraduate studies in Biology with specialization in Biochemistry, Biotechnology and Botany; Bachelor degree at Andhra University, Visakhapatnam, India

Work experience:

06/2010-10/2010	Research Assistant at the Department of Neurology, University Clinics of Bonn, Germany
12/2004-06/2006	Technical Assistant at the Department of Phytochemistry at FRLHT (Foundation for Revitalization of Local Health Traditions), Bengaluru, India

Training certificates:

09/2012	Seminar on Western blot techniques given by Merck Millipore
05/2004	10-days training on HPLC, GLC and UVspectrophotometer at IWST, Bengaluru, India

10. Publications

1) **Simma, N***, Bose, T*, Kahlfuß, S*, Mankiewicz, J, Lowinus T, Schraven, B, Heine, M, Bommhardt, U. NMDA-receptor antagonists block B-cell function but foster IL-10 production in BCR/CD40-activated B cells.
   Cell Communication and Signalling. 2014, 12 :75.

2) Kahlfuß, S*, **Simma,N***, Mankiewicz, J*, Bose, T*, Lowinus, T, Klein-Hessling, S, Sprengel, R, Schraven, B, Heine, M, Bommhardt, U. Immunosuppression by N-Methyl-D-aspartate receptor antagonists is mediated through $K_v1.3$ and $K_{Ca}3.1$ channels in T cells. *Molecular and Cellular Biology*. 2014, 34(5): 820-31. Selected as MCB spotlight paper

3) Pierau, M*, Na, SY*, **Simma, N**, Lowinus, T, Marx, A, Schraven, B, Bommhardt, UH. Constitutive Akt1 signals attenuate B-cell receptor signalling and proliferation, but enhance B-cell migration and effector function.
   European Journal of Immunology. 2012, 42(12): 3381-93.

* first co-author

Poster presentations:

09/2014 44th Annual Meeting of the German Society for Immunology (DGfI), Bonn, Germany; Biology of B-Lymphocytes: “NMDA-receptor antagonists impair B-cell proliferation, migration and enhance an IL-10 regulatory B-cell phenotype”

09/2013 43rd Annual Meeting of the German Society for Immunology (DGfI), Mainz, Germany; Signal crosstalk, Signal transduction and Calcium signalling: “NMDA receptor antagonists: Effective modulators of T cell signalling and effector function”