Glucocorticoid-induced apoptosis in animal models of Multiple Sclerosis

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Abstract

Glucocorticoids (GCs), produced endogenously or from therapeutically administered drugs, .are highly potent anti-inflammatory and immunosuppressive agents. GCs exert influence on many cell types of the immune system and impact a plethora of processes such as cytokine production, leukocyte differentiation, migration and adhesion, apoptosis induction and changes in morphology. Those that are most relevant for the modulation of neuroinflammatory diseases, however, are still under debate. In this review we will elaborate on how GCs impact inflammatory responses in general and revisit the ambivalent role that apoptosis plays in animal models of multiple sclerosis (MS). We will discuss arguments that speak in favor or against an essential function of GC-induced apoptosis in neuroinflammation. We anticipate that a better knowledge of the mechanisms that GCs employ will eventually find its way into clinical practice for the future benefit of afflicted patients.

1. Introduction

GCs are used to treat a plethora of disease entities ranging from autoimmunity and atopy to hematopoietic cancer. Nonetheless, it is not yet clear which of their mechanisms are indeed relevant to achieve a therapeutic benefit in each disorder. For example, it has been known for a long-time that GCs are potent inducers of apoptosis and it is therefore reasonable to believe that this mode of action plays a central role in many diseases. However, there is a great diversity among cell types and pathomechanisms being targeted by GCs and recent evidence suggests that the induction of apoptosis is less important than was previously thought. The emergence of novel modes of GC action including directed polarization of T lymphocytes, macrophages and dendritic cells, morphological alterations and redirection of lymphocyte migration, suggests that apoptosis induction may not always be mandatory for an improvement of disease symptoms. Currently, this notion is also discussed for multiple sclerosis (MS) and its animal model, experimental autoimmune encephaolmyelitis (EAE). High-dose pulse therapy with the synthetic GC methylprednisolon is a mainstay in the treatment of MS and various animal studies revealed that apoptosis occurs in response to such treatment. Nonetheless, there is also the observation that EAE can be ameliorated without GCs inducing cell death. Thus, we are now in the process of elucidating which of the hitherto described modes of GC action are critically required for the treatment of neuroinflammatory diseases, and whether this knowledge may assist in the development of more refined therapies that, under optimal conditions, even lack some of the side effects known to accompany GC administration to patients. Importantly, GC therapy has to stand up against new and innovative treatment regimens such as monoclonal antibodies and thus only a profound knowledge of its mechanisms will help to continuously improve this regimen.

2. Mechanisms of GCs in the control of the immune system

2.1. GCs and their receptors

The GC receptor (GR) is a member of the nuclear receptor superfamily. The 777 amino acid protein (in humans) was initially purified in the early 1980s and the cDNA cloned shortly afterwards.[1-3] The GR is composed of three major domains including a N-terminal transactivation domain, the central DNA-binding domain (DBD) and the C-terminal region (LBD) harboring the ligand binding pocket and a second transactivation motive.[4] In unstimulated cells the GR is found in the cytosol, sequestered in a heat shock protein complex.[5] Upon hormone binding, the GR dissociates from the heat shock protein complex and translocates into the nucleus facilitated by two nuclear localisation motifs. The GR recognizes and binds to conserved palindromic GC response elements (GRE) found in the promoter and enhancer region of a variety of genes and then homodimerization occurs.[6] This leads to the recruitment of coactivators such as CBP/p300, interaction with RNA polymerase II and initiation of transcription.[7] Although this mechanism was the first one to be discovered, several others have subsequently been indentified. First of all, the GR can bind to other transcription factors without contacting DNA itself.[8] Initially, this regulatory principle was demonstrated for the GR interacting with AP-1[9] but later extended to the GR also interacting with other transcription factors including NF-κB, CREB and Stat3. In most cases such tethering interactions result in transrepression by the GR, but occasionally they can also lead to synergistic transactivation, as in the case of Stat5.[10] A long time ago it was recognized that the GR is additionally able to repress gene transcription directly after binding to negative GREs (nGREs), but this mechanism was believed to be the exception rather than the rule.[11] Just recently, however, such nGREs were identified in several thousand genes across the whole genome, suggesting that this is indeed an important mechanism.[12] Finally, cytosolic interactions of the GR were reported to impact signal transduction by a number of pathways. These include cross-talk of the GR with PI3K,[13] PLC,[14] FAK,[15] and several components of the TCR complex such as Lck and Fyn.[16]

Irrespective of the molecular mode of action of the GR, its major ligand is GC's, a class of steroid hormones produced by the adrenal gland. The major GC in humans is cortisol whereas rodents predominantly produce corticosterone. In addition, a plethora of synthetic derivatives have been developed over the years which are characterized by improved potency or altered receptor specificity, thus being superior when administered to patients during therapy.[17] Finally, inactive variants such as cortisone can be converted into the active hormone by the help of 11β-hydroxysteroid-dehydrogenase type 1, which is expressed in a tissue-specific manner.[18]

GCs can also bind to the mineralocorticoid receptor (MR) which is highly homologous to the GR both at the cDNA and protein level.[19] It is larger than the GR but similarly structured, and encompasses 984 amino acids (in humans). After ligand binding, the MR translocates into the nucleus and recognizes the same palindromic GRE sequences as the GR.[19] Importantly, neither tethering interactions with other transcription factors nor repression via nGREs have so far been reported for the MR.[20] Although GCs are able to activate the MR this occurs only in a few cell-types. Instead, the predominant ligand of the MR is aldosterone. The reason for this is that 11β-hydroxysteroid-dehydrogenase type 2 converts GCs into inactive metabolites and thereby prevents them from binding to the MR.[21] Consequently, GCs activate the MR, only in cell types where this enzyme is absent, and within the immune system this only applies to macrophages.[22] Here it was observed that GCs interact with both the GR and MR although they exert opposing effects via each of the receptors. How the different transcriptional effects are achieved, in particular since both receptors bind to the same GREs, remains elusive.

2.2. Physiological regulation by GCs

Production and secretion of GCs is tightly controlled by the hypothalamus-pituitary-adrenal (HPA) axis.[23] Mostly neuronal stimuli, but also inflammatory mediators, elicit secretion of corticotropin releasing hormone (CRH) from the hypothalamus, which in turn induces

secretion of ACTH by the anterior pituitary.[24] Eventually this leads to the release of GCs from the adrenal cortex. A negative feedback loop ensures homeostasis by inhibiting the HPA axis both at the level of the hypothalamus and the pituitary.

The importance of GCs was most impressively demonstrated by the generation of GR knock-out mice.[25] The absence of the GR led to the death of the mutant mice within a few hours after birth due to lung failure. Owing to its ubiquitous expression, the GR and, more generally, GC actions are involved in a variety of developmental and physiological processes. Originally GCs were recognized for their role in controlling carbohydrate, lipid and protein metabolism. This function is part of the stress response initiated by GCs and prepares the body to cope with various external and internal challenges. More specifically, GCs increase gluconeogenesis and lipolysis, cause fat redistribution as well as glycogen breakdown and induce protein mobilization from skeletal muscle.[26-28] Another target organ of GCs is the brain, and deletion of the GR in mice was found to influence behaviour, memory formation and anxiety.[29, 30] From a practical point of view, the GCs' effects on the immune system are probably the most important ones. Stress accompanied by elevated GC levels has been known for many decades to result in immunosuppression. Prompted by this fact, Hench and colleagues in the 1940s were the first to trial GCs as a therapy for autoimmunity.[31] The treatment of rheumatoid arthritis (RA) patients with cotisone turned out to be highly effective and stimulated the development of novel GC derivatives with improved characteristics to treat inflammatory conditions. These include not only autoimmune diseases, but also various atopic disorders such as asthma and contact dermatitis.[32] In addition, GCs are currently widely used to prevent transplant rejection, suppress graft-versus-host-disease (GvHD) and as therapy for hematopoietic cancer.[33, 34] Of note, different mechanisms appear to be responsible for the therapeutic effects of GCs in the treatment of each of these diseases, and obtaining a deeper insight therein may help to develop more specific therapies in the future. In view of the ubiquitous expression of the GR, it is not unexpected that treating inflammatory disorders with GCs is often accompanied by unwanted side effects such as muscle wasting, osteoporosis and type II

diabetes.[28, 35, 36] For this reason, new approaches are being developed that aim to separate the adverse effects from the beneficial ones, although none of these have made it into clinical practice yet.[37]

2.3. The diversity of immunomodulatory GC effects

GCs exert a plethora of effects on the immune system. Essentially all cellular components of the hematopoietic system are under the control of GCs including macrophages, dendritic cells (DCs), granulocytes and lymphocytes.[38] Moreover, these cells's features can be influenced in multiple ways. GCs can impact the development and differentiation of immune cells as well as the balance between individual subtypes. GCs also modulate production of cytokines, chemokines, nitric oxid (NO), prostaglandins and cytotoxic molecules, and they are potent inducers of apoptosis in some, but not all, hematopoietic cell types. Finally, there is evidence that GCs alter morphological features of leukocytes and impact their migration and distribution within the body.

2.4. Impact of GCs on macrophages, granulocytes and dendritic cells

Macrophages are found in a variety of tissues where they contribute to innate immunity after ligation of pattern recognition receptors such as TLRs as well as to adaptive immunity by serving as antigen-presenting cells (APCs). Macrophages produce cytokines such as TNFα, IL-1β and IL-6 and produce cytotoxic molecules like NO. GCs suppress production and release of the aforementioned molecules mostly through tethering interactions of the GR with NF-κB, AP-1 and IRF3.[39-41] In addition, GCs control features of macrophages that are part of the adaptive immune system. They down-regulate MHC class II molecules required for T cell priming[42] and repress IL-12 which directs T cell development towards the Th1 phenotype. More recently, profound effects of GCs on macrophage differentiation were recognised. These cells can adopt at least two different phenotypes called M1 and M2.[43]

Whereas the former one is induced by LPS and IFNγ and is pro-inflammatory in nature, the latter results from the influence of immunosuppressive agents such as IL-4, IL-10 or GCs.[44] The phenotype that macrophages adopt under the influence of GC exposure is designated M2c and is characterized by an up-regulation of the scavenger receptor CD163 and the mannose receptor CD206.[44] Liposome-encapsulated GCs are particularly potent in inducing the M2c phenotype, while GC effects mediated by the MR seem to have an inhibitory effect on M2c differentiation.[45, 46] Since this particular phenotype is characterized by improved wound-healing and phagocytosis, it was implicated in tissue repair and thought to have a beneficial effect on inflammatory responses *in vivo*.

Neutrophil granulocytes are components of innate immunity and form the first barrier against infection. They are attracted to the site of inflammation by chemokines and infiltrate tissues by means of adhesion molecules. Integrins expressed by neutrophils and their receptors on endothelial cells are inhibited by GCs, which leads to reduced extravasation.[47] GCs additionally suppress cytokine production resulting in impaired effector functions. Importantly, GCs do not induce granulocytes apoptosis, which is in sharp contrast to their strong pro-apoptotic effect on lymphocytes.[48]

DCs are professional APCs that serve to take up foreign antigen mostly at peripheral sites such as the skin and subsequently prime T cells in secondary lymphoid organs. GCs impede DC function by down-regulating MHC class II molecules and inhibiting migration of these cells to the draining lymph nodes.[49] Similar to macrophages, GCs not only modulate the function of DCs but also their differentiation. That said, GCs were found to induce a tolerogenic phenotype characterized by suppression of T cell priming and induction of regulatory T (Treg) cells. One such site in which this occurs is the placenta, where GCs tolerize DCs in conjunction with other hormones, thereby preventing rejection of the MHC-missmatched embryo.[50]

2.5. Impact of GCs on T and B lymphocytes

Reports over recent years have claimed that GCs influence thymocyte development, as well as positive and negative selection, through their ability to induce apoptosis.[51, 52] Nonetheless, these effects are controversial. In contrast, it is undoubted that mature T cells in peripheral lymphoid organs are influenced by GCs in many ways. On the one hand they impact on the differentiation of T cells into either a Th1 or Th2 phenotype.[53, 54] The former one is responsible for cellular immunity and serves to activate macrophages, while the latter one fosters humoral immunity by providing B cell help. Thereby GCs indirectly impact the type of immune response mounted which has important implications for autoimmunity.[54] In contrast, to date little is known about the role of GCs in controlling Th17 responses which are involved in the pathogenesis of autoimmune diseases such as MS.[55, 56] Besides their capacity to modulate T cell differentiation, GCs also suppress many effector functions of T cells including the secretion of cytokines such as IL-2, IFNγ and TNFα as well as expression of adhesion molecules like LFA-1 and VLA-4.[56-58] Furthermore, GCs potently induce T cell apoptosis, although individual subsets of thymocytes and peripheral T cells differ in their susceptibility to GC-induced cell death (GICD).[59] A longstanding debate in the field concerns the impact of GCs on Treg cells. Whilst several reports suggested that GCs lead to the expansion and improved function of Treg cells,[60, 61] more recent experiments have demonstrated unaltered Treg cell numbers in mice under normal conditions as well as in the context of neuroinflammation.[56, 62] Moreover, administration of GCs to human volunteers in the absence of an ongoing inflammatory disease did not result in elevated numbers of Treg cells.[62] Thus, at present it is unlikely that Treg cells are crucial mediators of the immunosuppressive activity of GCs.

Several lines of evidence indicate that GCs impact the T cells' distribution within the body. Experiments in a mouse model of MS revealed that administration of GCs prevented T cells from infiltrating the CNS.[56] This might have been caused by down-regulation of adhesion molecules, as mentioned above, but could also be due to an altered responsiveness of T cells to chemokine signaling as reported in the literature for the control of CXCR4 by GCs.[63]

Furthermore, GCs are potent modulators of T cell morphology, which impacts the cells' functional features.[64] Shortly after exposure to GCs, the cytoskeleton of effector T cells becomes rearranged, leading to a rapid loss of polarization. Consequently, the effector T cells are no longer able to efficiently migrate or interact with APCs. This rapid effect occurs independently of translation but requires the GR, is mediated by phosphorylation of ERM proteins which fulfill an important function in shaping T cell morphology, and seems to involve a PLC-dependent pathway.[64] The same cytoskeleton rearrangements were noted *in vivo* suggesting that the observed process plays an important role in inflammatory responses and their resolution by GCs.

Compared to T cells much less is known with regard to the impact of GCs on B cells.[65] It was reported that after GC treatment, B cell numbers were reduced due to the induction of apoptosis and that there is a shift from IgG to IgE production. However, whether the latter observation was a direct effect on B cells or rather caused by the enforced Th2 differentiation under these conditions remains to be explored.

3. Pathways to apoptosis

3.1. Mechanisms of cell death induction and execution

Programmed cell death or apoptosis is an important process both during embryonic development and homeostasis of adult tissue.[66] Apoptosis can be executed by two distinct, but ultimately converging pathways: the so-called ′extrinsic′ and ′intrinsic′ apoptotic pathways. While the former one is induced upon ligation of membrane bound receptors,[67] the latter one is activated in response to intracellular stress, such as DNA damage or oncogene activation.[68] The ligation of cell surface receptors, such as CD95 (FAS), TRAIL or TNF in the ′extrinsic′ apoptotic pathway, leads to the formation of the Death Induced Signaling Complex (DISC) at the Death Domains (DD) of the receptor. In the initial phase of the ′extrinsic′ apoptotic pathway, pro-caspase-8 is recruited via the adaptor protein FADD

or TRADD into the DISC and activated, and can in turn activate the executioner caspase-3. Finally, active caspase-3 is able to cleave and thereby inactivate many cellular proteins leading to the demolition and killing of the cells.[67]

The ′intrinsic′ or mitochondrial pathway of apoptosis is mainly regulated by members of the Bcl-2 protein family.[68] All Bcl-2 family members show sequence homology in their ′Bcl-2 homology regions′ (BH domains), and they can be divided into two major subgroups: anti- and pro-apoptotic factors.[69] The pro-apoptotic proteins can be further divided into the ′BH3 only′ and the Bax/Bak like members. Upon induction of the ′intrinsic′ apoptotic cascade, the ′BH3 only′ proteins (Bim, Bad, Noxa, Puma, Bid, Bmf, Hrk) are induced and able to activate the Bax/Bak like members either (i) indirectly through binding and thereby neutralization of pro-survival Bcl-2 family members (A1, Bcl-2, Bcl-w, Bcl-X_L and Mcl-1) bound to Bax/Bak[70] or (ii) directly by binding to Bax/ Bak.[71, 72] Either way, active Bax/Bak proteins oligomerize to form pores into the outer mitochondrial membrane leading to the release of apoptogenic factors, such as cytochrome C into the cytosol.[73] Cytochrome C binds together with ATP to the adaptor protein Apaf1, leading to the activation of caspase-9. [74] Active caspase-9 in turn activates effector caspase-3, -6 and -7, culminating in the decay of the cell.

3.2. The role of apoptosis in the immune system

Apoptosis is important for the proper development and maintenance of hematopoietic cells.[75] For example, apoptosis can be initiated during lymphopoiesis, because developing T and B cells either fail to express a productively rearranged antigen receptor, or produce a receptor with too strong affinity for self-peptides. These selection processes help to prevent autoimmunity.[76] It is worth mentioning that the killing of developing lymphocytes is solely dependent on the `intrinsic` and not the ′extrinsic` pathway. Of note, recent reports suggested that the main inducer of apoptosis in developing T and B cells is the `BH3 only` protein Bim and to a lesser extent the ‘BH3 only’ protein Puma.[77]

Apoptosis is not only important for the development but also the homeostasis of lymphocytes after the termination of an adaptive immune response. During the shutdown of an immune response, B and T cells are removed by activation-induced cell death (AICD). Early studies concluded that the `extrinsic` apoptotic pathway triggered by CD95/FAS was the main inducer of AICD. However, it was recently shown that the `BH3 only` protein Bim is one of the major components and assists CD95/FAS in this process.[78] The removal of activated B and T cells after the immune response is a crucial mechanism to reduce autoimmune disorders.

3.3. The mechanism of GICD

GC-induced apoptosis (GICD) requires both the GR and its transactivation domain. The induction of GR-mediated killing is through the ′intrinsic′ apoptotic pathway and linked to *de novo* gene expression.[27] The importance of the 'intrinsic' apoptotic pathway was confirmed in mice, in which the transgenic expression of the pro-survival protein Bcl-2 in the hematopoietic lineage rendered these cells completely resistant to GICD.[79, 80, 81] Accordingly it was shown that the knockout of the Bcl-2 gene in mice led to enhanced cell death of thymocytes *in vitro* in response to GC treatment.[82] Other pro-survival Bcl-2 family members implicated in GICD are Mcl-1 and A1 and to a lesser extent Bcl-x_L.[83-85] However, these studies were mostly performed in cell lines and further validation in mouse models is required.

As mentioned before, GICD depends on *de novo* gene synthesis and the upregulation of many target genes.[86-89] However, functionally only the pro-apoptotic Bcl-2 family members Bim and Puma were found to be important. This notion is based on the observation that Bim- or Puma-deficient lymphocytes showed a lower apoptosis rate than wildtype cells when exposed to the synthetic GC analogue dexamethasone *in vivo*.[90] Interestingly, the GR does not directly induce Bim transcription in GICD, but indirectly through either (i) up-regulation of c-Jun and Runx2, which positively regulate Bim transcription, or (ii) down-

regulation of the miR-17-92 cluster known to inhibit Bim mRNA translation.[91-94] Nonetheless, these studies are entirely based on *in vitro* data and further efforts to validate them *in vivo* are needed. Currently, the regulation of Puma in GICD is not very well understood and further experiments are therefore recommended. Besides Bim and Puma, no other pro-apoptotic Bcl-2 family member has to date been implicated in GICD.

4. Animal models of MS

4.1. Experimental autoimmune encephalomyelitis (EAE)

EAE is a widely employed animal model of MS, a neurological disease of presumed autoimmune origin.[95] MS most frequently affects young adults in Western Europe and Northern America and is characterized by leukocyte infiltration into the CNS, which leads to inflammation, focal demyelination, neuronal loss and increasing disability of afflicted patients.[96] Many aspects of the human disease can be mimicked in animal models although none of them recapitulates all features of the complex human disorder.[95] Therefore multiple variants of EAE have been developed over the years, each model permitting the analysis of specific features of MS. In most cases, EAE is induced by immunizing mice or rats with CNS antigens such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) or proteolipid protein (PLP) together with a strong adjuvant. Alternatively, preactivated pathogenic T cells can be transferred to induce the disease[97] or, in specific cases, the disease may also arise spontaneously as a consequence of genetic manipulation.[98] Depending on the mouse or rat strain the disease course differs between individual EAE models. Induction of EAE in Lewis rats using MBP or by adoptive transfer of encephalitogenic T cells in the same strain results in a monophasic disease course mainly reflecting the inflammatory features of MS.[54, 97] In contrast, immunization of C57Bl/6 mice with MOG peptide 35-55 leads to a chronic diseases that involves demyelination and neuronal damage.[56, 99] More closely mimicking the predominant form of the human disease,

namely relapsing-remitting MS, are DA rats immunized with MOG protein[100] or SJL mice immunized with PLP[101]. In all case, the disease involves infiltration of antigen-specific $CD4^+$ T cells into the CNS after breaching of the blood-brain barrier (BBB). These cells mostly belong to the Th1 and Th17 subtypes producing IFNγ, IL17A and IL17F as well as GM-CSF.[102, 103] The resulting pro-inflammatory milieu enforces an additional influx of leukocytes into the CNS including more $CD4^+$ T cells but also bystander cells such as monocytes and macrophages, B lymphocytes, granulocytes, Treg cells and $CD8^+$ T cells. Whilst $CD4^+$ T cells are widely considered to be the main pathogenic cell type in most EAE models, it became clear over the years that $CD8^+$ T cells are possibly as important.[104] However, their exact function is still controversial.

4.2. The ambivalent role of apoptosis in neuroinflammation

Apoptosis plays multiple roles in EAE.[105] It was initially detected in the spinal cord of Lewis rats suffering from EAE and found to primarily affect T cells shortly before disease remission.[106, 107] Consequently, apoptosis is thought to be responsible for the resolution of inflammation in the monophasic EAE model but also during relapsing-remitting EAE. Conversely, resident and infiltrating leukocytes cause massive oligodendrocytes apoptosis[108] and thereby contribute to the pathology seen in neuroinflammatory disorders. Oligodendrocytes form the myelin sheet around axons and are responsible for its maintenance and repair. Consequently, oligodendrocyte death is detrimental for neuronal function and is a primary cause of the disease symptoms seen in the chronic phase of some EAE models.

Mediators of the ‘extrinsic’ and ‘intrinsic’ apoptotic pathway fulfill various functions in EAE.[105] Analysis of the ‘extrinsic’ pathway was stimulated by the availability of two naturally occurring mouse mutants named *lpr* (lacking for Fas) and *gld* (lacking FasL). Both strains are largely resistant to EAE induction irrespective of the antigen used for immunization.[109] Reciprocal adoptive transfer experiments served to define the cell types in which each of

the two molecules was essential. Full-blown disease was only observed when Fas was present on host cells while FasL had to be expressed by antigen-specific T cells.[110] Interestingly, oligodendrocytes are the only cell-type in the CNS expressing significant levels of Fas, which is further up-regulated during neuroinflammation.[111] In contrast, the infiltrating T cells as well as activated microglia are the primary producers of FasL in EAE.[112] Taken together, it seems that FasL expressing cells cause massive damage to oligodendrocytes through induction of apoptosis and thereby contribute to the manifestation of the disease. Nevertheless, the 'extrinsic' pathway is also involved in the resolution of EAE.[110, 113] More precisely, FasL-expressing cells are able to induce apoptosis of infiltrating antigen-specific T cells and thereby presumably contribute to the remission and thus clinical improvement of the disease in later phases.

The 'intrinsic' pathway of apoptosis is particularly relevant in leukocytes during EAE.[105] Insight into its role has mostly been obtained by analyzing mice with targeted mutations of various Bcl-2 family members. Overexpression of the anti-inflammatory Bcl-2 protein in T cells did not impact on EAE in the initial phase.[114] Instead, it led to a more severe chronic phase, which was most likely the consequence of reduced "beneficial" apoptosis taking place in the CNS. The same observation was made for mice overexpressing Bcl-x_L, although these animals additionally showed an earlier onset of EAE.[115] These two observations highlight that apoptosis induction by the 'intrinsic' pathway is involved in shaping the chronic phase of the disease. Genetic disruption of individual pro-apoptotic molecules is more complex. Bax-deficient mice are partially protected against EAE although the cell types responsible for the observed phenotype remain unclear.[116] Bim-deficient mice were completely refractory to EAE induction, but in this case the phenotype did not seem to be related to impaired apoptosis but rather to defective T cell activation and cytokine production.[117] Interestingly, our unpublished results indicate that Puma knock-out mice do not exhibit the same phenotype with regard to EAE, although both molecules fulfill highly related functions.

5. Relevance of GICD in animal models of MS

5.1. GICD of T lymphocytes during EAE

Induction of T cell apoptosis by GCs occurs both in secondary lymphoid organs and the CNS and has been described for different EAE models.[56, 118] As outlined above, challenges such as inflammation activate the HPA-axis, which results in the release of endogenous GCs including corticosterone. According to the current notion elevated GC levels lead to the induction of apoptosis primarily in T cells, which contributes to the remission of disease symptoms, for example in the monophasic EAE model in Lewis rats.[119] Accordingly, adrenalectomized Lewis rats develop a fatal EAE accompanied by low levels of apoptosis.[120] Nonetheless, it is unclear whether reduced apoptosis is relevant for the aggravated disease course after adrenalectomy or whether it is only a bystander effect. Similarly, C57Bl/6 mice that lack the GR in either T cells or the entire hematopoietic system suffer from an aggravated chronic EAE.[56] Again it is not known whether the worsened disease course is due to the inability of GCs to induce T cell apoptosis or their inability to cause other functional changes in T cells.

High-dose GC administration is the standard therapy to treat acute disease bouts in MS patients.[121, 122] Such regimens can be mimicked for example in the AT-EAE model in Lewis rats. In this scenario application of GCs also ameliorates the disease, which is accompanied by T cell apoptosis in the CNS.[118, 123] On the other hand, treatment of chronic EAE in C57Bl/6 mice with the synthetic GC dexamethasone did not result in GICD in the CNS but rather in peripheral lymphoid organs.[56] Although it was not clear in this model whether apoptosis induction was relevant for the therapeutic effect, the data argue against GICD occurring *in situ* in the CNS.

Collectively, it is undoubted that endogenous and therapeutic GCs induce T cell apoptosis during EAE and that this correlates with improved clinical symptoms. However, whether this has to occur in the CNS itself or rather in peripheral lymphoid organs, and whether it is at all causally linked to the beneficial effects of GCs is still enigmatic.

5.2. Arguments against an essential role of GICD of T cells in EAE

The first observation that challenged a major role of GICD in EAE concerned the finding that application of a low dose of GCs ameliorated the disease despite the lack of apoptosis induction.[124] This suggested that additional mechanisms must at least contribute to the beneficial activity of GCs in neuroinflammation.

Liposome-encapsulated GCs were reported to possess higher efficacy in the treatment of EAE as compared to free GCs.[125] This was amongst others assigned to their preferential uptake by macrophages. Although GCs are believed to mainly target T cells during EAE, analysis of liposomal prednisolon revealed that this formulation was able to ameliorate EAE by polarizing macrophages towards the anti-inflammatory M2 phenotype without the need to impact T cells.[45] Thus, encapsulation of GCs into liposomes apparently alters their mode of action. This, however, suggests that T cell apoptosis cannot be the only essential mechanism of GCs in EAE interference.

Compound A (CpdA) is a non-steroidal ligand of the GR that belongs to the class of so-called dissociating GCs.[126] These drugs are able to repress gene transcription through DNA-binding-independent tethering mechanisms of the GR (see above) while they do not induce gene transactivation. Treatment of EAE with CpdA ameliorated EAE at certain dosages and, most importantly, at these concentrations induction of T cell apoptosis by CpdA was not observed.[127] It is noteworthy that this finding is compatible with the previous observation made in genetically manipulated mice, that GICD requires gene transactivation by GR DNA-binding.[27] Consequently, induction of T cell apoptosis does not appear to be essential for the beneficial effect of GCs in EAE.

In T cells, the enzyme acid sphingomyelinase (aSMase) is involved in mediating exocytosis of vesicles, cytokine secretion and induction of apoptosis.[128, 129] In addition, aSMase is specifically required for protection of effector memory T cells from GICD.[130] In double-positive thymocytes and naïve peripheral T cells GCs efficiently induced apoptosis

irrespective of whether aSMase was present. Effector memory T cells that are normally resistant to GICD, however, partially lost their protection against GICD, presumably due to impaired IL-2 secretion that normally serves to upregulate Bcl-x_L.[130] The enhanced sensitivity of effector T cells towards GICD neither impacted EAE nor did it interfere with the capacity of GCs to treat the disease.[130] This supports the notion that GICD, at least of effector T cells, is not an essential mechanism of GC action in EAE.

Further support for the notion that modulation of effector T cells by GCs is not essential for disease intervention came from results obtained in an AT-EAE model in Lewis rats.[123] When the GR was inactivated in antigen-specific effector T cells by stable expression of siRNAs, they became refractory to GICD as well as to transactivation and transrepression by the GR in general. Nonetheless, AT-EAE induced in GR-deficient or wildtype cells was indistinguishable with regard to disease severity, remission and treatability by GCs.[123] Consequently, effects of endogenous and therapeutic GCs on antigen-specific effector T cells are insufficient for intervention with neuroinflammatory diseases such as EAE and therefore GICD of effector T cells can only be of minor importance.

Collectively, T cell apoptosis undoubtedly occurs in the course of EAE and correlates with increased levels of endogenous GCs or application of therapeutic GC derivatives. However, it appears that GICD, especially of effector T cells, does neither determine disease initiation nor progression or responsiveness to high-dose GC therapy. Hence, other effects of GCs are presumably more relevant in the context of EAE.

6. Perspective

There is good evidence that T cell apoptosis occurs after GC treatment of mice and rats, but whether this mechanism is indeed essential for ameliorating EAE is doubtful. This notion has potentially important implications. For example, GC derivatives that are unable to induce apoptosis but still capable of mediating other effects of GCs such as altered migration or morphology should be promising candidates for new drugs to be tested in MS

intervention. Namely, such compounds could have advantages over currently available ones. Most notably, the severity of side effects might be reduced since apoptosis induction by GCs is know to require the transactivation function of the GR that is also essential for some of the adverse GC effects. Moreover, new compounds may also overcome resistance to GC treatment, as frequently developed by patients after repeated application of the drug during relapse. Collectively, we would hope that a detailed understanding of how GCs act in MS, based on the analysis of animal models such as EAE, will pave the way for alternative compounds, drug formulations and treatment regimens in the future.

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Acknowledgements

We would like to thank Dr. Gemma Kelly for language correction. This work was supported by grants from the *DFG* (Re1631/7-1, Lu634/8-1, SFB-TRR 43 / B13), Leukaemia Foundation, Australia and NHMRC Project Grant (APP1049720) for MJH. The authors have no conflicting financial interests.

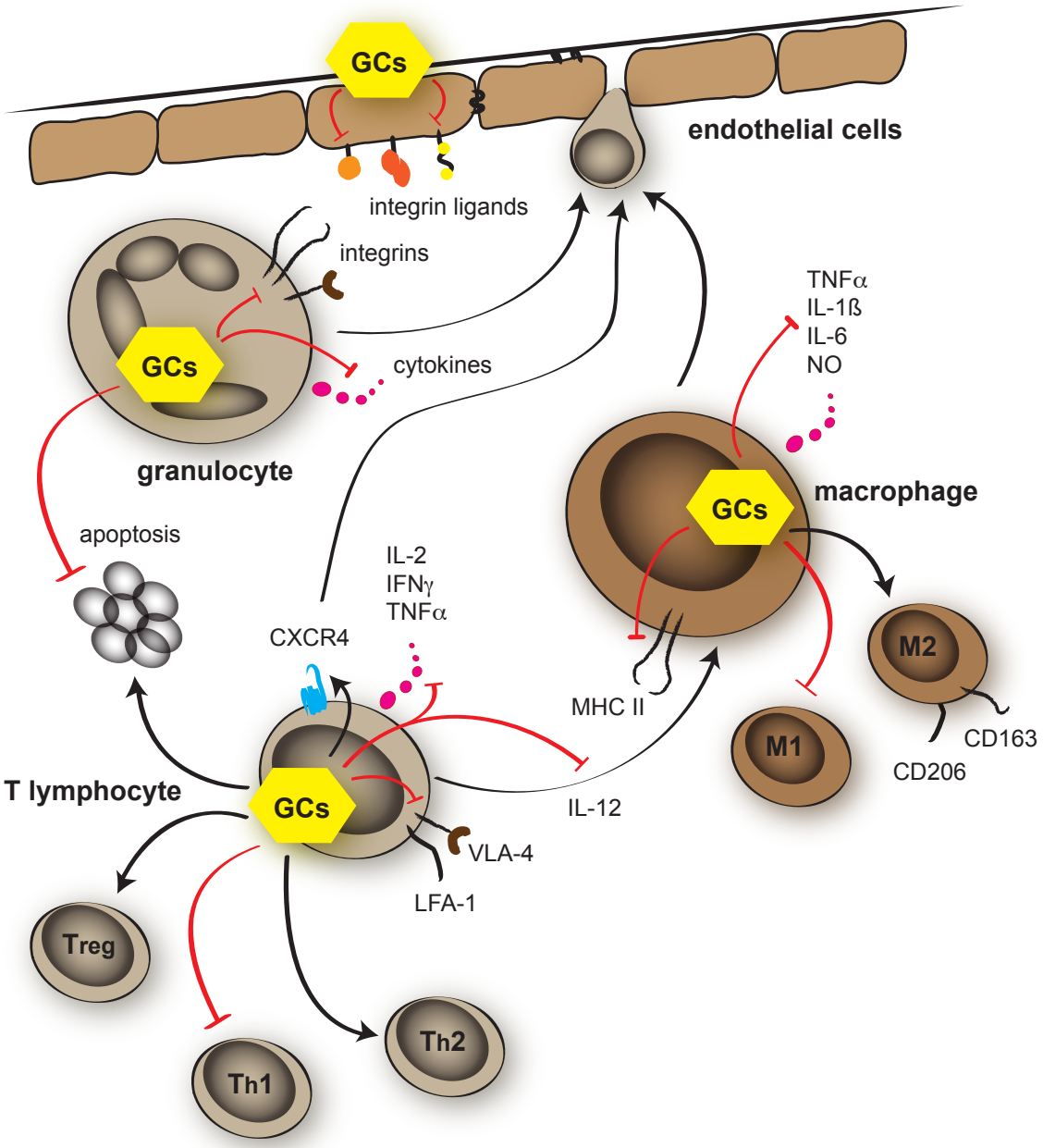
Figure legends

Figure 1. Impact of GCs on selected leukocyte subsets. GCs suppress production of cytokines and nitric oxid (NO) by macrophages and down-regulate MHC class II surface expression. In addition, GCs favor macrophage polarization towards the anti-inflammatory M2 phenotyp characterized by surface expression of CD163 and CD206. In contrast, M1 polarization is inhibited. In neutrophil granulocytes GCs suppress production of cytokines and expression of integrins. T lymphocytes are influenced by GCs in multiple ways: cytokines including IL-2, IFNγ and TNFα are suppressed, the chemokine receptor CXCR4 is up-regulated and surface levels of integrins such as LFA-1 and VLA-4 are reduced. In addition, the capacity of T cells to activate macrophages is diminished via suppression of IL-12 production. GCs also favor polarization of T cells towards the Th2 phenotype whereas Th1 differentiation is inhibited. Another major effect of GCs is induction of T cell apoptosis. In contrast, a potential positive influene of GCs on Treg cells is controversial. Migration of all types of immune cells across endothelial barriers is inhibited by GCs by down-regulation of integrins as well as of their ligands expressed on endothelial cells.

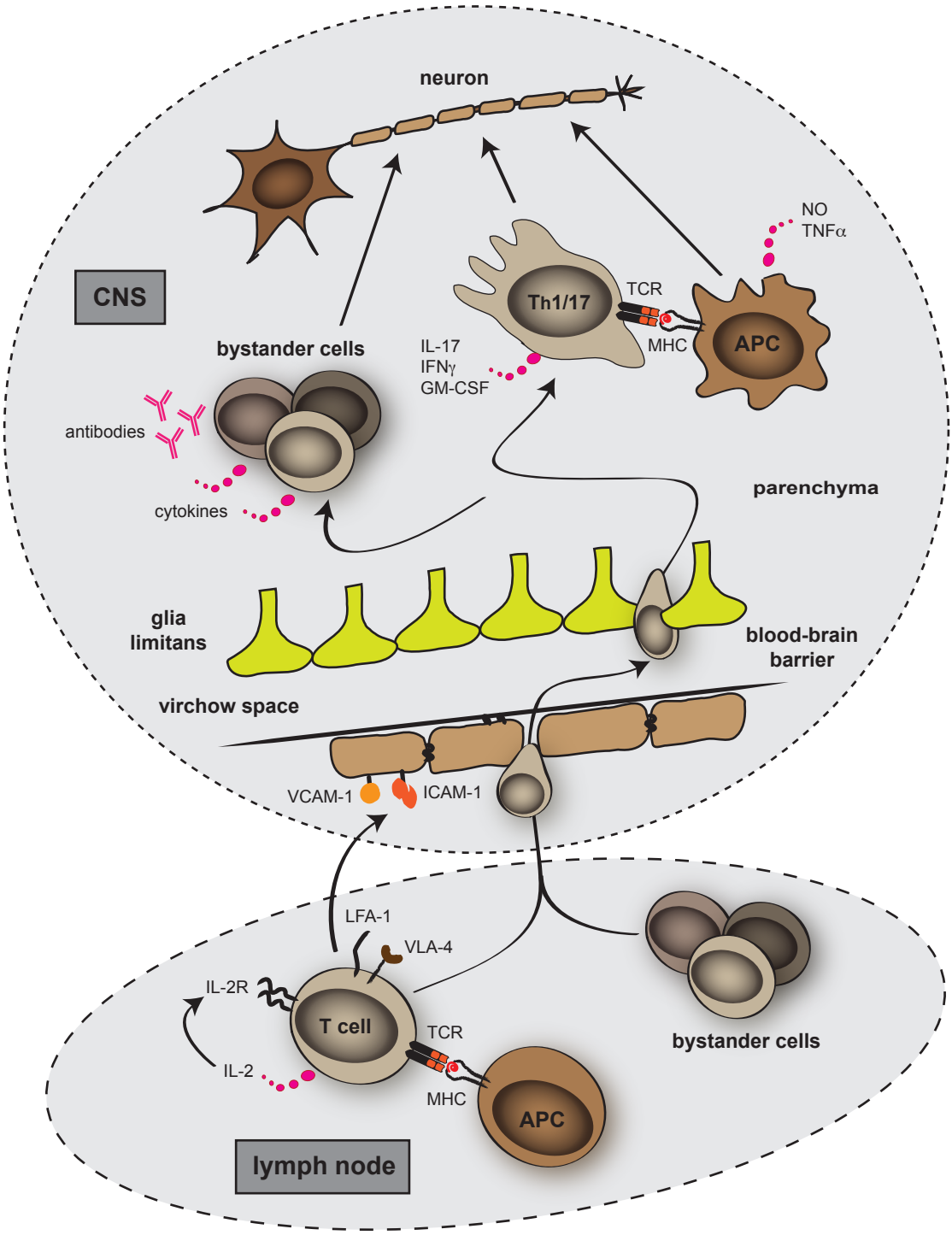
Figure 2. Pathomechanism of EAE. In the lymph nodes T cells become activated by antigen-presenting cells (APCs) following interaction of the T cell receptor (TCR) with MHC-peptide complexes. Secretion of IL-2 and its subsequent binding to the IL-2 receptor (IL-2R) cause T cell expansion in an autocrine manner and induce integrin expression. Interaction of LFA-1 and VLA-4 with their respective ligands ICAM-1 and VCAM-1 on endothelial cells of the blood-brain barrier then allows T cell infiltration into the CNS parenchyma across the virchow space and the glia limitans. Finally, T cell reactivation by CNS-resident APCs such as microgial cells leads to the secretion of pro-inflammatory cytokines including IL-17, IFNγ and GM-CSF by differentiated antigen-specific Th1 and Th17 cells. Conversely, microglial cells are activated by pathogenic T cells and release TNFα and nitric oxid (NO).

Due to the impaired integrity of the blood-brain barrier a variety of bystander cells including T and B lymphocytes as well as myeloid cells enter the CNS and release cytokines and antibodies. In combination, infiltrating leukocytes and their mediators attack neurons and oligodendrocytes and eventually cause axonal damage.

Figure 3. Mechanisms of GC-induced apoptosis. GCs passively enter the T cell and bind to the glucocorticoid receptor (GR) in the cytosol. Following translocation into the nucleus the GR dimerizes upon recognition of GC response elements (GREs) present in the promoter and enhancer regions of a variety of genes. Amongst others, Puma and Bim are up-regulated through direct and indirect mechanisms. Bim up-regulation is mediated by transcriptional activation of c-Jun and Runx 2 and down-regulation of the miR17-92 cluster through global inhibition of the microRNA machinery. Subsequently, Puma and Bim activate oligomerization of Bax and Bak located in the outer mitochondrial membrane leading to the release of cytochrome c (Cyt c), which activates the apoptosome consisting of Apaf1 and caspase 9 (Casp 9). Caspase 3 (Casp 3) is then activated leading to the cleavage of apoptotic substrates, which eventually results in T cell death.



Herold and Reichardt, Figure 1



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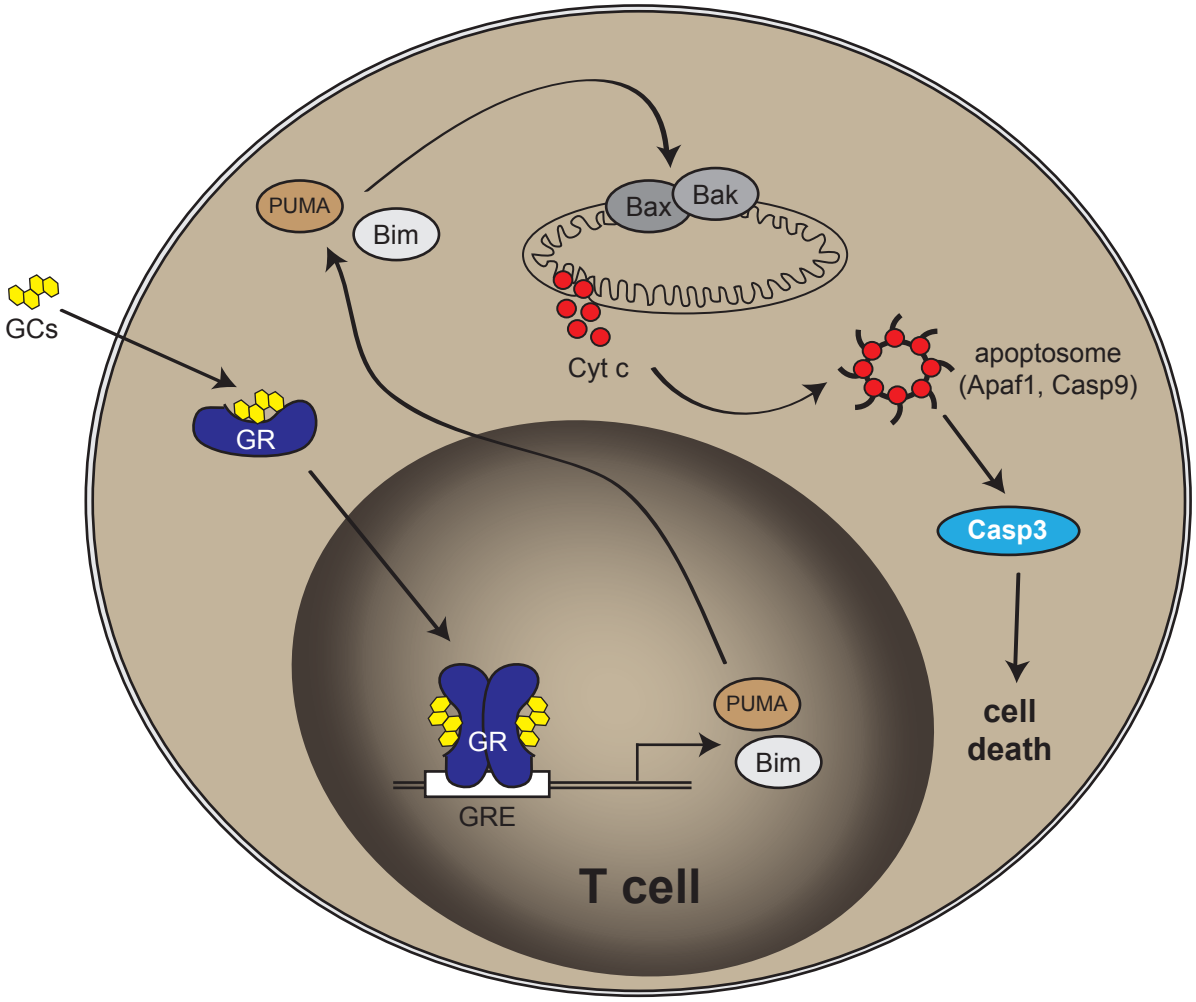

Herold and Reichardt, Figure 3