

Accepted Manuscript

Title: Pharmacological targeting of allergen-specific T lymphocytes

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PII: S0165-2478(17)30088-3

DOI: <http://dx.doi.org/doi:10.1016/j.imlet.2017.03.010>

Reference: IMLET 6009

To appear in: *Immunology Letters*

Received date: 25-2-2017

Accepted date: 15-3-2017

Please cite this article as: Tauber Peter A, Pickl Winfried F. Pharmacological targeting of allergen-specific T lymphocytes. *Immunology Letters* <http://dx.doi.org/10.1016/j.imlet.2017.03.010>

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Pharmacological targeting of allergen-specific T lymphocytes¹

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¹ 6-mercaptopurine, 6-MP; activator protein 1, AP-1; adrenergic receptor agonist, ARA; adrenergic receptor, AR; altered peptide ligands, APL; aminoimidazole carboxamide ribonucleotide transformylase, AICART; aryl hydrocarbon receptor, AhR; cyclic adenosine monophosphate, cAMP; chemokine ligand, CCL; chronic myelogenous leukemia, CML; cyclo-oxygenase 2, COX2; cytosolic GR, cGR; dendritic cell, DC; deoxy-6-thioguanosine 5' triphosphate, dGS; di-hydrofolate reductase, DHFR; experimental autoimmune encephalomyelitis, EAE; Fcε receptor, FcεR; FK506 binding protein, FKBP; glucinamide ribonucleotide transformylase, GART; glucocorticoid receptor, GR; granulocyte macrophage colony stimulating factor, GM-CSF; heat-shock protein 90, Hsp90; histamine receptor, HR; histidine decarboxylase, HDC; histone deacetylase, HDAC; IκB kinase, IKK; immuno-receptor tyrosine based activation motif, ITAM; interferon, IFN; interleukin, IL; janus kinase 3, JAK3, JUN N-terminal kinase, JNK; knock-out, ko; leukotriene receptor, LTR; leukotriene, LT; cysteinyl, Cys; mammalian target of rapamycin, m TOR; membrane GR, mGR; methotrexate, MTX; mineralocorticoid receptor, MR; mycophenolate mofetil, MMF; mycophenolic acid, MPA; inosinic acid dehydrogenase, IMPDH; nuclear factor of activated T cells, NFAT; peripheral blood mononuclear cell, PBMC; peripheral blood, PB; phorbol-myristate-12-acetate, PMA; phytohemagglutinin, PHA; prostaglandin E, PGE; protein kinase A, PKA; protein kinase C, PKC; purified protein derivative, PPD; retinoic acid receptor, RAR; retinoic acid, RA; retinoid x receptor, RXR; rheumatoid arthritis, RA; S-methyl-thioinosine 5'-monophosphate, 6-Me-Thio-IMP; selective glucocorticoid receptor agonist, SEGRA; SHIP1, specific immunotherapy, SIT; staphylococcal superantigen B, SEB; T cell receptor, TCR; thiopurine methyltransferase, TPMT; thyroid hormone receptor, THR; toll-like receptor, TLR; tumor necrosis factor, TNF; tyrosine kinase, TK; vitamin D receptor, VDR; ζ-chain associated protein 70, ZAP70.

PICKL, highlights

- Classic anti-allergic and anti-asthmatic drugs frequently target Th cell responses
- Allergic patients might benefit from classic immunosuppressive drugs other than GC
- Novel targeted drugs impact on allergen-specific Th cell function

Abstract

Allergic disorders are the result of a complex pathophysiology, involving major cellular lineages and a multitude of humoral factors of the innate and adaptive immune system, and has the tendency to involve multiple organs. Consequently, even standard pharmacological treatment of allergies is rarely specific but usually targets more than one pathway/cellular system at a time. Accordingly, many of the classic anti-allergic drugs have a critical impact also on T helper cells, which are pivotal not only during the sensitization but also the maintenance phase of allergic diseases. Recent years have seen a dramatic increase of novel drugs with the potency to interfere, more or less specifically, with T lymphocyte function, which might, possibly together with classic anti-allergic drugs, help harnessing one of the central cellular players in allergic responses. A major theme in the years to come will be a thoughtful combination of previously established with recently developed treatment modalities.

Keywords: Anti-allergic drugs, T cells, allergy, pharmacological modulation, immunomodulation.

1. Introduction

This review will focus on the T cell-directed effects of classic anti-allergic drugs and on established and novel immunomodulatory drugs, which have an intrinsic potential to interfere with undesired allergen-specific T cell reactions and offer treatment options especially in severe manifestations of allergic diseases. Since the first synthesis of histamine more than 100 years ago, the first clinical application of cortisone for auto-inflammatory and allergic diseases more than 60 years ago and the development of the first immuno-suppressive substances targeting calcineurin, such as Cyclosporin A and Tacrolimus (FK506) in the 1970ies and 1980ies respectively, the armamentarium of both classic anti-allergic and immunosuppressive drugs has grown steadily, nowadays ranging from targeted drugs impacting on very defined signaling pathways to the therapeutic application of oligonucleotides and monoclonal antibodies [1-4]. The T cell modulatory potential of classic anti-allergic drugs such as histamine receptor antagonists, β 2-adrenoreceptor agonists, leukotriene receptor antagonists and corticoids will be discussed and contrasted with classic and novel immunosuppressive drugs. To restrict the scope of this review to a manageable volume, we here concentrate on the pharmacological options to interfere with allergic diseases but we refrain from discussing the multiple beneficial aspects of allergen-specific immunotherapy (SIT) and of altered peptide ligands (APL) on allergen-specific T lymphocytes.

2. Principle mode of action and effects on T lymphocyte function of classical anti-allergic drugs

This section describes the impact on T helper cells of systemically applied classic anti-allergic drugs but omits chromoglicic acid-based mast cell membrane stabilizers, which are almost exclusively applied locally.

2.1. Histamine and anti-histaminic drugs

Histamine, is a tissue hormone and neurotransmitter, which is ubiquitously present in the human body (with highest expression in the lung, skin and gut) and has been characterized already in 1907 by *Windaus* and *Vogt* [5]. Later, it was characterized by *Dale* and *Laidlaw* as a bodily substance in humans [6]. Already in 1937 *Bovet* and *Straub* developed first substances, which functioned as anti-histamines spearheading anti-histamine research and also the development of first neuroleptics [7]. Histamine is clearly one of the important mediators of allergic inflammation. Within the immune system, histamine is stored in mast cells and basophils from where it gets released either upon FcεR-mediated triggers, engagement of the anaphylatoxin receptors C3a and/or C5a or other basic substances (e.g. bradykinin, substance P, mastoparan and others) resulting in immediate type allergic reactions, resulting in smooth muscle contraction and increased vascular permeability. Histamine is also produced in non-immune cell types at high levels such as enterochromaffine cells in the stomach, lymph nodes and thymus. Genetic ablation of histidine decarboxylase (HDC) leading to complete absence of histamine resulted in a reduction of the overall numbers of mast cells and the appearance of abnormal mast cells but neither influenced viability nor fertility of mice [8]. However, HDC ablation had a major impact on the sleep-wake cycle of animals which were accompanied with changes in the cortical-electroencephalograms [9]. As of today four different 7-span transmembrane and G-coupled receptors termed histamin-1-receptors (H1R) to H4R have been characterized (**Fig. 1**). Of

note, these receptors reveal constitutive activity in the absence of agonists, which gets shifted by inverse agonists representing the classic 'anti-histamines'. While the H3R is almost exclusively expressed in the brain, the other HRs are variably expressed on different leukocyte populations [10]. Apart from mast cells and basophils also CD4 Th cells express a whole set of different HRs. Consequently, histamine has been shown to differentially regulate lymphocyte responses [11]. Engagement of the H1R leads to IFN- γ production and thus enhanced Th1 cell responses. In contrast, engagement of the H2R, which is predominantly expressed on Th2 cells, leads to suppression of Th1 and Th2 responses [12]. This fits to the observation that T cells of H2R ko mice produce increased levels of both Th1 and Th2 signature cytokines [13]. Compatible with these findings, ablation of either of the two receptors alters the susceptibility of mice to experimental allergic encephalomyelitis [14]. This clear dichotomy between H1R and H2R is, however, discussed somewhat controversially in other reports, since it has been shown that engagement of H1R on T cells of rhinitic subjects enhanced Th2 cytokine production in one study, while it has the opposite effects in controls individuals and asthmatics [15]. Apart from T cells, H2R-signaling exerts its inhibitory effects also on antigen presenting cells in which a whole collection of cytokines get inhibited [16, 17].

The function of the H4R on T cells, which was identified by genomic homology screening much later than the other three receptors, is less well defined [18]. Recent investigations point to a role H4R in the induction and/or aggravation of pruritus in atopic dermatitis. *In vitro* polarized Th2 cells, which clearly express the H4R on the mRNA and protein level, up-regulated mRNA for IL-31 upon H4R ligation. IL-31 represents a pruritogenic cytokine [19]. Notably, in patients suffering from atopic dermatitis, H4R tends to be expressed at elevated levels and stimulation of PBMC of such patients with an H4R ligand and in the presence of staphylococcal superantigen B (SEB) resulted in higher IL-31 levels. The molecular mechanism for H4R function seems to be related to the signal transducer molecule AP-1,

which mediates and is required for the expression of Th2 related cytokines IL-4, IL-5 and IL13. In line with these findings, H4R agonists have been shown to inhibit PPD-induced IFN- γ production of PBMC [20]. Of note, H4R antagonists such as JNJ-7777120 should strongly impact on Th cell functions including those of Th17 cells [21] and should be of benefit for the treatment of allergic symptoms. In fact, *Cowden et al.* have shown that treatment with a H4R antagonist reduces airway hyperreactivity along with the production of the Th2 cytokines IL-5 and IL-13 [22]. In contrast, H4R engagement was shown to reduce the production of inflammatory cytokines produced by plasmacytoid dendritic cells, such as TNF- α , IFN- γ and CXCL8 [23].

However, the situation seems to be more complicated, due to the pleiotropic functions of histamine since it has been shown in the past that H4R engagement by the *bona fide* H4R agonist 4-methyl-histamine, *e.g.* by intra-tracheal instillation, can also lead to the accumulation of Treg cells in lung tissues, which revealed increased IL-10 production [24]. In contrast, H4R agonists or histamine itself fosters Th17 responses on the mRNA but also protein level since H4R is expressed on Th17 cells [25]. The impaired migratory and regulatory function of Treg might also be the reason for the increased susceptibility of H4R ko mice to EAE [26]. Due to its modulatory activity on CCR2 expression, H4R also impacts on the circulation of both Th cells but also antigen presenting cells such as epidermal dendritic cells and monocytes [27, 28]. Due to the anti-inflammatory action of novel H4R antagonists, such as JNJ-7777120, but also due to their anti-pruritic activity, these substances represent prime drugs for the treatment of contact hypersensitivity [29]. Moreover, a more general regulatory role of H2R and H4R has been shown in $\gamma\delta$ T cells, with H4R increasing intracellular Ca^{2+} levels, actin polymerization and chemotaxis, while H2R leads to cAMP accumulation and down-regulates cytotoxicity against tumor target cells [30].

2.2. β 2 adrenergic-receptor agonists

β 2-adrenergic receptor agonists (β 2-ARA) are important drugs asthma therapy [31]. While the primary intention for β 2-ARA application in asthmatic patients are certainly their immediate bronchodilatory effects, subtle effects of β 2-ARA on immune cells have been described. In fact, β 2-AR have been shown to be widely expressed on different subsets of leukocytes, their expression on T cells has also been clearly demonstrated [32-34]. While some studies suggested that β 2-AR are differentially expressed on different Th cell subsets, with clear-cut expression on Th1 cell clones, but rather low to non-detectable expression on Th2 cell clones [32], others have shown that β 2-AR are also regularly expressed and functional on Th2 cells [35]. Consequently, also the effects on T cells of β 2-AR engagement have been controversially discussed during the last years depending on the responder T cell population studied and the stimuli used to activate them. Notably, some reports showed that β 2-ARA monotherapy might increase the number of Th2 cells. By stimulating PB lymphocytes of healthy individuals or lupus patients with IL-2 in the presence of isoproterenol, a significant accumulation of Th2 cells producing IL-13 was observed in one study [36]. This effect was attributed to the PKA-activating properties of the G-protein coupled β 2-AR [36]. In contrast, isoproterenol inhibited the growth of CD3/CD28 stimulated T cells, which was also accompanied by a reduction in the secretion of Th2 cytokines in another study [37]. Accordingly, β 2-ARA treatment might increase cytokine production and secretion by type 2 T cells [36, 38] upon antigen-independent but not necessarily upon antigen-dependent T cell stimulation. In contrast, other studies investigating the effect of the β 2-ARA salbutamol on PHA plus PMA treated PB T cells of healthy subjects did not reveal inhibitory effects when compared to dexamethasone, which significantly suppressed the production of IL-4 and IL-5 and to a lesser degree also of IFN- γ [39].

Thus, studies investigating the effects on T cells of β 2-ARA used as mono-substances have led to seemingly controversial results, which might, however, be explainable by the mode of

cellular activation applied. However, the situation becomes much clearer when β 2-ARA were combined with steroids and their combined effects were monitored. Along those lines, *Loza et al.* found that β 2-ARA in combination with the steroids budesonid, tended to inhibit the generation of IL-13 producing Th2 cells in PB lymphocytes of asthmatics, while the numbers and percentages of IFN- γ producing cells remained unchanged [38]. This also fits to the observation by others that β 2-ARA synergize with glucocorticoids in inducing enhanced production of IL-10 by human regulatory CD4⁺ T cells [40]. In the clinical setting this would indicate that dose escalation with long-acting β 2-ARA might translate into additional clinical benefit in previous moderate responders to inhaled glucocorticoids and β 2-ARA. Along those lines, allergen-specific T cells lines induced in the presence of fluticasone and salmeterol led to the generation of Tregs, which inhibited IL-5 and IL-13 production by Th2 cells when co-cultured at a ratio of 4:1 between effector and regulatory T cells [40]. This inhibition was clearly IL-10 dependent since it could be blocked by anti-IL-10 receptor antibodies. Consequently, β 2-ARA might not only lead to bronchodilatation but, especially when combined with steroids, also seems to be proficient in inhibiting the production and release of effector cytokines from Th2 cells. Mechanistically, the activity of β 2-AR is governed by the G_s-coupled anenylyl cyclase system leading to a rise in intracellular cAMP followed by an increase in PKA activity [41]. In fact, elevation of β 2-AR signaling has been shown to induce Th2 cells with increased IL-4 production [42, 43].

On top of the subtle cues regulating its activity, functionally relevant polymorphisms within the β 2-AR system have been described recently. Especially the haplotype β 2-AR (2/4) has been identified to be associated with severe asthma and poor bronchodilatator response [44]. Whether this polymorphism also affects signaling and function in immune cells remains to be shown in the future. Nevertheless, it is tempting to speculate that polymorphisms in β 2-AR might impact on the generation of Treg cells, as discussed above, and might also differentially

impact on other Th cell subtypes and thus contribute to the increased susceptibility to asthma and its exacerbation in different populations [44]. Apart from allelic variants, recent evidence suggests that the expression of β 2-AR in Th1 and Th2 cells might also be regulated by epigenetic cues [43]. In fact, Th2 culturing conditions resulted in decreased β 2-AR gene expression. This decrease is likely brought about by lower levels of H3 and H4 acetylation and H3K4 methylation, which are accompanied by higher levels in H3K9 and H3K27 methylation [43].

2.3. Muscarinic AChR

Apart from the above-described neurotransmitters, lymphocytes have been shown to express muscarinic cholinergic receptors [45, 46]. In the Jurkat T cell line several muscarinic agonists, such as acetylcholine, carbachol, muscarine and oxotremorine-M, induced a transient elevation of cytosolic Ca^{2+} concentrations, which was insensitive to pertussis and botulinus toxin, *i.e.* not dependent on GTP-binding proteins [47], but inhibitable by phorbol ester triggered PKC activation. Notably, T cells of nasal polyposis samples released TNF- α upon exposure to increasing doses of methacholine [48].

2.4. Leukotrienes and leukotriene receptor-antagonists

Lipoxygenase-dependent arachidonic acid metabolites such as leukotrienes (LT) get synthesized by a large number of inflammatory cells, thrombocytes and reticulocytes [49, 50]. The different cell types characteristically express different sets of lipoxygenases and thus produce a cell-type specific spectrum of LT [51, 52]. Notably, many inflammatory stimuli amplify the production of cysteinyl LTs (LTC_4 , LTD_4 and LTE_4), which induce bronchoconstriction and mucus secretion in the lungs and inhibit tracheal mucus transport [53]. Two cysteinyl LT receptors with differential expression characteristics have been described in the past, Cys-LTR1, which is expressed in peripheral blood leukocytes, lung

macrophages and airway smooth muscle cells, and Cys-LTR2, which is expressed in heart, adrenal glands, placenta and peripheral blood leukocytes [54]. A number of LT-specific antagonists for the treatment of asthma have been developed in the past [55]. While resting T lymphocytes express rather low levels of Cys-LTR1 and Cys-LTR2, they tend to up-regulate both receptors upon ligation of the TCR/CD3 complex with anti-CD3 mAbs [56]. Similarly, elevated Cys-LTR1 levels have also been observed on T cells of house dust mite allergic individual upon stimulation with Der p, which could be prevented by co-incubation with the Cys-LTR1 inhibitor MK571 [57]. Notably, co-incubation of anti-CD3 mAb treated T cells with the selective Cys-LTR1 inhibitor montelukast increased IFN- γ secretion but also specific cell death of T cells while leaving IL-4 secretion levels unaltered. The increase in T cellular death was attributed to the gradual loss of the anti-apoptotic molecule Bcl-2 [56]. A more recent study demonstrated that, in fact, the effect of Cys-LT on Th2 cells is much broader than originally estimated [58]. In fact, highly purified human Th2 cells isolated from buffy coats revealed enhanced LTE₄-dependent production of pro-inflammatory proteins CCL3, CCL3L1, CCL3L3 and CCL4L2 and together with PGE2 increased the expression of the non-classical Th2 mediators IL-22, IL-8 and GM-CSF [58]. Thus apart from their bronchodilatory effects, LTAs might also critically influence Th2 cell function.

2.5. Glucocorticoids: classical representatives of immunosuppressive drugs

Glucocorticoids regulate central aspects of cellular metabolism by driving gluconeogenesis, in addition, they exert strong anti-inflammatory activity. Cortisone, the first immunosuppressive drug for allergic conditions such as asthma and allergic dermatitis was already clinically applied in the 1950's [59, 60]. Since then, a large number of synthetic glucocorticoids with increased potency and low systemic burden when applied locally, *e.g.* in the airways, paved their way into the clinician's holster such as betamethasone, beclomethasone, budesonide and fluticasone [61].

Glucocorticoids exert pleiotropic effects via the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), both of which are expressed ubiquitously on different cell types [62-64]. With regard to the GR, the mechanisms of action can be divided into classical genomic effects mediated upon binding to the cytosolic GR (cGR) and non-genomic effects also mediated through the cGR and by binding to a putative membrane GR, described in a single report to be a G-protein coupled receptor [65, 66]. Notably, membrane expression of a GR (mGR) was also shown in one study in lymphocytes from leukemic patients and in a human T cell line [67]. Additionally, other reports suggested that glucocorticoids might also exert their function through non-specific interactions with the plasma membrane [66]. In principle, GRs reside in the cytosol where they are attached to a chaperone complex, which consist of heat-shock protein 90 (hsp90), hsp70, hsp90-binding protein p23 and immunophilins [68]. Upon binding of glucocorticoids to GRs, the such formed complexes translocate into the nucleus where they can either bind to glucocorticoid responsive elements (GRE) of the DNA, thereby facilitating or hindering gene transcription, or they directly interact with other transcription factors in an enhancing or suppressing fashion, like AP-1 or NF κ B [68]. These genomic processes are termed transactivation and transrepression, respectively [69].

The basic principles of the GR also apply to the MR with the exception that a membrane receptor for mineralocorticoids has also been suggested but not sufficiently proven as of yet [70]. Very similarly to the GR, also the MR usually resides in the cytoplasm and translocates to the nucleus after binding of its physiologic substrate, *i.e.* aldosterone and is also capable of mediating rapid non-genomic cellular changes [71, 72].

In T lymphocytes, glucocorticoids have been shown to inhibit T-bet transcriptional activity by facilitating direct interaction of the GR with T-bet and thereby preventing it from binding to the DNA. Apart from T-bet, GCs were also shown to inhibit GATA-3 activity via inhibition of protein-kinase A(PKA)-dependent phosphorylation of GATA-3 and p38 in murine

splenocytes [73]. Principally, glucocorticoid actions result in reduced expression of pro-inflammatory genes such as *e.g.* cyclo-oxygenase 2 (COX2) mainly via inhibition of NFκB but also stimulation of anti-inflammatory cytokines such as IL-10 [74-76].

Notably, Foxp3⁺CD25⁺ T cells from atopic and non-atopic individuals showed increased suppressive activity on allergen-stimulated effector CD4⁺CD25⁻ T cells via an IL-10 dependent mechanism when they were pre-incubated with fluticasone-propionate [77]. Along those lines, another study demonstrated that CD4⁺ T cells from asthmatic individuals receiving inhaled steroids or receiving inhaled plus systemic steroids had significantly increased mRNA expression of Foxp3 and IL-10 compared to asthmatic patients not treated with steroids or healthy individuals [78].

Thus, despite many other effects, GC treatment seems to favor regulatory T cell function while suppressing Th1 and Th2 cell differentiation and action. In addition, also a direct interaction between GCs and TCR signaling was suggested in one study that described rapid, immunosuppressive effects of dexamethasone pre-treatment on anti-CD3/CD28-stimulated human CD4⁺ T cells by GR-dependent inhibition of the lymphocyte-specific PTKs Lck and Fyn [79]. Mechanistically, this inhibitory activity of GCs was shown to be induced by their disruption of a complex formed between Lck, Fyn, Hsp90 and GR, which, under physiological conditions, associates with the TCR complex upon TCR ligation [80]. Apart from lymphocyte-specific PTKs, high-dose GC treatment also leads to phosphorylation of the tyrosine residues 315 and 492 of the ξ-chain-associated ZAP-70 protein and thereby account for further non-genomic inhibitory effects on the downstream TCR signaling cascade [81].

With regard to MCs, no direct effects on T lymphocytes upon ligand binding have been shown as of today. Nevertheless, MC treatment of mice led to a shift towards the Th1/Th17 cell lineage, however, this was due to indirect, agonistic effects by MR acting on dendritic cells (DCs) [82]. Notably, this Th17-promoting effects could be inhibited by co-treatment

with either one of the aldosterone antagonizing substances eplerenone or spironolactone underscoring the T cell-specific immunomodulatory capacity of MR [83].

Prolonged GC treatment leads to a number of adverse side effects such as lipodystrophy, reduced insulin sensitivity, osteoporosis, candidiasis and psychological alterations [62]. Another problem of GC-treatment is represented by primary or secondary resistance to GCs, representing a major limitation when f.i. approaching the patient suffering from allergic asthma [84]. GC-resistance was found to be associated with increased expression of c-Fos, phosphorylated c-JUN and phosphorylated JUN N-terminal kinase (JNK) in immune-histochemically analyzed skin punch biopsies from GC-resistant individuals when compared to GC-sensitive individuals with regards to tuberculin-mediated cutaneous responses. Consequently, failure of GC-mediated inhibition of JNK phosphorylation might explain GC resistance [85]. While certainly of interest, this study could, however, not show a T cell-specific difference in the degree of JNK phosphorylation or expression. That the JNK phosphorylation status might be critical for allergen-specific inflammation has been shown in another study in which inhibition of JNK by the ATP-competitive inhibitor SP600125, prior to allergen challenge, reduced the expression of toll-like receptor 9 (TLR9) and significantly reduced the number of inflammatory cells and ovalbumin-specific IgE in a mouse model of acute asthma [86]. This study is in accordance with earlier work, which had described overexpression of c-Fos in T cells from GC-resistant but not GC-sensitive asthmatics. Thus, activity of both c-Fos and c-JUN, fine-tuned by up-stream kinases, which form the AP-1 transcription factor complex seem to be dysregulated in allergy and associated with GC resistance [87]. Studies performed with T cells from healthy individuals using SP600125 revealed specific inhibition of IL-2, IL-13 and IFN- γ but not of the major IgE-class switching factor IL-4 upon stimulation with anti-CD3/B7-H2 coated beads [88]. This implies that interference with JNK signaling might have different effects in health and disease. In

summary, it will be interesting to study the effects of drugs targeting JNK or up-stream constituents particularly with regard to their impact on Th effector functions such as the cytokines produced by them and the transcription factors activated, which can be studied in much more detail with the technologies available as of today. Apart from regulating AP-1-dependent gene expression GCs have been shown to inhibit IL-4 by interaction with the transcription factor NFAT [89].

Due to the severe side effects when administered systemically, there is a strong need for corticoids that only exert selected glucocorticoid actions such as immunosuppression or attenuation of inflammatory reactions while minimizing unwanted side effects. With this objective in mind, the development of selective glucocorticoid receptor agonists (SEGRAs) was encouraged. Similar to the classic representatives also the newly developed selective glucocorticoid receptor agonists (SEGRAs) such as Mapracorat (ZK 245186), which currently undergo clinical evaluation [90] and have significantly fewer side effects (*e.g.* skin atrophy, hyperglycemia and hepatotoxicity), show clear-cut inhibitory effects on T cells, with are equipotent to their parent drugs [91].

3. Principle mode of action and effects on T lymphocyte function of classical immunosuppressive drugs

This section describes immunosuppressants used to treat severe forms of allergic diseases and their impact on T lymphocyte function.

3.1. Immunosuppressive drugs interfering with DNA metabolism

Azathioprine:

Azathioprine is a pro-drug of 6-mercaptopurine (6-MP) and works by means of its various metabolites, *e.g.* deoxy-6-thioguanosine-5'-triphosphate (dGS) and S-methyl-thioinosine-5'-monophosphate (6-Me-Thio-IMP) through different mechanisms by inhibition of *de novo* synthesis of purines, protein synthesis and by induction of apoptosis [92]. The inhibitory capacity of purine analogues with a 6-mercapto group on the growth of *Lactobacillus casei* was first described by *Elion et al.* in 1951 [93]. The relative selectivity of these drugs on lymphocytes was explained by their exclusive inhibition of *de novo* synthesis of purines, which represents the major route for providing nucleotides in lymphocytes compared to other cell types that can also sufficiently make use of the purine salvage pathway [94]. Care has to be taken with regard to the inter-individual differences in the activity of the enzymes thiopurine-methyltransferase (TPMT) and NUDT15 when treating patients with thiopurines, since both enzymes are important for inactivating cytotoxic metabolites [95]. Furthermore, the combination of allopurinol, a xanthine oxidase inhibitor, together with mercaptopurines can have hazardous effects due to the inhibition of the conversion of 6-MP to the metabolite thiouracil acid [96]. Apart from limiting the purine pool, azathioprine metabolites, such as 6-thioguanine triphosphate, seem to directly affect T lymphocyte signaling, *f.i.* by converting the co-stimulatory signal provided by CD28 ligation into a pro-apoptotic signal in human CD4⁺ lymphocytes by its interference with Rac1 via 6-Thio-GTP formation which replaces GTP and abrogates Rac1 downstream signaling [97, 98]. While short-term *in vivo* treatment with thiopurines already blocks lymphocyte proliferation but still preserves their effector

functions, only long-term treatment induces depletion of memory cells, which have a history of multiple antigen encounter [99]. The situation seems to be slightly different when activated (transformed) T cell types were interrogated, since one study found that Azathioprine also inhibited the up-regulation of inflammatory genes such as α 4-integrin, TNFRSF7 and TRAIL in Jurkat T lymphocytes upon stimulation [100]. According to a meta-analysis in the Cochrane database, the application of Azathioprine as a corticosteroid sparing agent in asthma is lacking evidence for support due to the low numbers and types of studies available [101]. Nevertheless, with regard to atopic dermatitis, systemic TPMT-adjusted monotherapy with azathioprine appeared to be a useful alternative to systemic glucocorticoids in patients with symptoms insufficiently controlled by topical treatment [102].

Methotrexate:

Methotrexate is a folic acid analogue with minimal structural differences inhibiting the enzyme dihydrofolate reductase (DHFR) [103]. The activity of folic acid analogues against different neoplasms and the ability to induce partial remission in acute leukemia in humans by the analogue 4-amino-N¹⁰-pteroylglutamic acid (Aminopterin) was first described by *Farber* in the 1940's [104, 105].

In animal studies Amethopterin (Methotrexate, MTX) showed a ten times lower cytotoxicity than Aminopterin which may explain why it became the clinical folate analogue of choice before newer folate antagonists such as i.a. Pemetrexed or Raltitrexed were designed [106, 107]. Due to the inhibition of DHFR, folate metabolites are generated which, together with MTX, exert their antineoplastic and immunosuppressive effects via further mechanisms such as inhibition of thymidylate synthase, aminoimidazole carboxamide ribonucleotide transformylase (AICART) and glycinamide ribonucleotide transformylase (GART), enzymes also involved in the nucleotide synthesis pathway [108]. Since mammalian cells are not able to synthesize folic acid *de novo* and thus dependent on external folate supply, Methotrexate

treatment affects many different cell types and thus exerts considerable side effects, especially in frequently dividing cells, such as epithelial cells in the gut and cells of hematopoietic origin, such as immune cells. While rapidly dividing cells which depend on *de novo* purine synthesis are more prominently affected MTX treatment, MTX also exerts additional immunomodulatory effects by inhibition of cytokine production, DNA and protein methylation and histone and protein acetylation [109]. Notably, the clinical success of MTX treatment in a collective of RA patients has been shown to significantly positively correlate with high numbers of CD39⁺CD4⁺Treg and consequently high levels of ADO production [110]. Mechanistically, this is thought to be caused by MTX-mediated demethylation of the *Foxp3* promoter, which has been shown to be also responsible for increased CTLA-4 expression on CD4⁺ T cells of patients undergoing MTX treatment [111]. Several studies, among them also a double-blind randomized placebo controlled study showed that low doses of MTX can be an effective steroid-sparing treatment for patients with steroid-dependent asthma and that overall tolerance was very good [112, 113]. This effect is also reflected by the fact that *ex vivo* MTX treatment increases the effects of glucocorticoids on T cells from asthmatic patients [114].

Mycophenolate mofetil:

Mycophenolate mofetil (MMF) is a prodrug of mycophenolic acid which was approved for the treatment of kidney transplant patients in the United States already in 1995 [115].

Mycophenolic acid (MPA) was first isolated from *Penicillium stoloniferum* and described for its antibiotic effects [116, 117]. Soon its potential as an anti-cancer drug became apparent and later on it was also evaluated for its capacity as an immuno-suppressant [118, 119]. The main mechanism of action of MPA is the inhibition of inosinic acid dehydrogenase (IMPDH) and thereby the inhibition of purine nucleotide synthesis [120]. Human IMPDH is expressed in two isoforms whereby MPA is more potently inhibiting the type II isoform [121]. As outlined

for thiopurines, interference with the *de novo* purine synthesis is supposed to affect lymphocytes more prominently than other cell types. Some authors suggested an additional selectivity for lymphocytes due to the increased expression of the type II isoform of IMPDH in stimulated T cells [122]. However, one study using PMA-stimulated PBMCs showed the upregulation of both isoforms and reasoned that type I and II IMPDH are responsible for nucleotide synthesis in activated T cells [123]. It was also shown that MPA, by inhibiting the *de novo* guanine nucleotide synthesis, resulted in a cell cycle block affecting the transition from G0 to S phase of activated normal human T cells and that inhibition of cyclin D/CDK6 kinase induction was responsible for this effect [124]. That MPA also alters the epigenetic status of CD4⁺ T cells by modulating HAT and HDAC function has been shown recently [125]. The application of MMF for patients with severe therapy-resistant asthma was described in a case study of 22 patients in which more than half of the patients benefitted from the treatment [126]. In an earlier study, the successful treatment of a patient suffering from non-allergic, therapy-resistant asthma with MMF had been reported [127]. Also, for severe cases of atopic dermatitis, which were not adequately controlled by topical treatment, MMF was successfully applied [128]. However, a definitive recommendation as to whether or not favor MMF compared to other immunosuppressants cannot be given as of yet due to the lack of randomized, placebo controlled clinical trials for this indication [129].

3.2. Calcineurin targeting drugs, classic immunosuppressive drugs

Calcineurin is a phosphatase responsible for the de-phosphorylation of nuclear factor of activated T cells (NFAT) [130]. Dephosphorylated NFAT can enter the nucleus and promote transcription of genes such as IL-2, facilitating T cell activation and proliferation [131]. Tacrolimus (FK-506) and cyclosporin A but also rapamycin (Sirolimus) are ligands of the so-called immunophilins, *i.e.* cyclophilin in the case of cyclosporine A and FK506-binding protein (FKBP) with regard to FK-506 (tacrolimus) and sirolimus (Rapamycin) [132]. These

immunophilin-drug complexes then mediate inhibition of calcineurin, which results in impaired de-phosphorylation and nuclear translocation of NFAT [133, 134]. Although its name might suggest T cell specific expression, the transcription factor NFAT also exerts regulatory functions in other tissues such as the central nervous system, blood vessels, the heart and the kidneys [135]. This may also explain the pleiotropic side effects, which are associated with calcineurin inhibitor treatment, such as nephrotoxicity, neurotoxicity (*i.e.* tremor, headache, paresthesia) and metabolic disturbances such as dyslipidemia and diabetes [136].

Immunosuppressive drugs characterized by a macrolide structure, such as Sirolimus (Rapamycin), also use FKBP to form an active complex [133, 134], however, they exert their function by inhibiting the serine/threonine-kinase activity of the mammalian target of rapamycin (mTOR). Consequently, the activity of the downstream target of mTOR, *i.e.* p70S6 gets impaired resulting in reduced activation of S6 and thus less activation of p34cdc2 kinase which is critical for cell cycle progression and thus proliferation. Interestingly, also other macrolides and macrolide-related compounds such as the antibiotics clarithromycin and azithromycin have been described to exert immunomodulatory effects [137]. In one study azithromycin inhibited proliferation and cytokine production of human CD4⁺ T cells upon stimulation with anti-CD3/anti-CD28 coated microbeads [138]. Notably, these effects were shown to be mediated by mTOR in an FKBP-independent fashion suggesting a direct interaction between azithromycin and mTOR [138]. Apart from its application in the post-transplant setting Cyclosporin has also a place as a corticosteroid-sparing drug in individuals suffering from corticosteroid-dependent asthma, *i.e.* individuals suffering from asthma and requiring continuous corticosteroid medication [139]. Furthermore, its application as an aerosol in asthmatic patients has been tested and was well-tolerated and, in fact, novel galenic formulations for this route of administration are under development [140, 141]. Along those lines also entirely cell-nonpermeable analogs of CsA, MM218, have

been developed, with the intention of blocking extracellular immunophilins functioning as important chemotactic agents among other cells types for CD4⁺ T cells [142]. In a mouse model of allergic asthma MM214 inhibited lung inflammation by 80% and directly inhibited the recruitment to the lungs of allergen-specific CD4⁺ T cells [142].

While no placebo-controlled clinical study investigating the application of Tacrolimus in patients suffering from asthma could be found occasional case reports describes the successful achievement of control over severe corticosteroid-dependent asthma with Tacrolimus [143]. Of note, however, there is evidence for increased occurrence of allergic sensitizations among transplant patients treated with Tacrolimus but with Cyclosporine A [144-146]. Whether these findings are due to pharmacodynamic or kinetic differences of the two drugs remains to be shown in future studies. More established is the use of tacrolimus and the structurally related pimecrolimus for the treatment of atopic dermatitis for mild to moderate disease activity, a treatment protocol, which has been already approved for children from 2 years onwards [147].

4. Principle mode of action and effects on allergen-specific T lymphocytes of selected vitamins known to impact on T cell function

4.1. Vitamin A (Retinoic acid)

Vitamin A and its derivatives such as the metabolite alitretinoin (9-*cis*-retinoic acid) and isotretinoin (13-*cis* retinoic acid) are commonly used for the treatment of different dermatological conditions such as *acne vulgaris* and chronic hand eczema while all-*trans* retinoic acid (t-RA) is used to treat pro-myelocytic acute leukemia [148-151]. Several intracellular receptors have been identified as binding partners for alitretinoin such as the retinoic acid receptor (RAR) and the retinoid x receptor (RXR), both representing nuclear receptors, the latter one being relatively specific for alitretinoin and not being bound by some other vitamin A derivatives, *e.g.* all-*trans* retinoic acid (t-RA) [152]. The action of retinoids via RAR or RXR requires heterodimerization of RAR with RXR or homodimerization of RXR and translocation into the nucleus although these two types of dimers bind to different DNA response elements [153]. Furthermore, RXR also binds to and functionally interacts with the vitamin D receptor (VDR) and the thyroid hormone receptor (TR) thereby stimulating DNA binding upon encountering their respective ligands [154].

Interestingly, vitamin A deficiency drives naïve CD4⁺ T cells towards the Th1 phenotype whereas treatment with alitretinoin can polarize these cells into Th2 cells *via* the RXR, both processes being independent of antigen presenting cells [155]. This was confirmed in another study in human PBMCs and purified human T cells in which t-RA and alitretinoin treatment resulted in increased mRNA expression levels of IL-4 and IL-5 as well as decreased expression of IFN- γ [156]. The same study also showed that t-RA treatment decreased expression of the Th1 master regulatory transcription factor T-bet but increased the Th2 lineage specific transcription factor GATA-3. Another study found that human peripheral blood T cells express the RAR isoforms α and γ but not β and that treatment of these cells with alitretinoin inhibited phytohemagglutinin (PHA) induced proliferation *via* ligation of

RAR- γ and inhibition of janus kinase 3 (JAK3) expression [157]. Also, an anti-apoptotic effect of retinoid X receptor agonists such as alitretinoin on naïve CD4⁺ T cells *via* up-regulation of Bcl2a1 was described [158]. Moreover, Tóth *et al.* showed that interaction of an RAR α /RXR heterodimer upon binding of alitretinoin interacted and altered the transcriptional activity of the ligated GR enhancing apoptosis of mouse thymocytes *in vivo*, which was independent of DNA binding of RAR or RXR [159]. The potential Th2-promoting effect of retinoic acid derivatives was confirmed in the follow-up of a randomized placebo-controlled trial investigating the effects of neonatal vitamin A supplementation, which was associated with an increased risk of atopy and wheeze in females but not boys [160].

All these findings raise the critical question whether retinoic acid derivatives could be created that, similar to the SEGRAs described above, would exert selected agonistic and antagonistic effects thereby altering T helper cell polarization towards a desired phenotype.

4.2. Vitamin D (1,25-Dihydroxycholecalciferol, Calcitriol):

Similar to the GR or the RAR also the vitamin D receptor (VDR) constitutes a nuclear receptor [161]. The vitamin D response elements either bind the VDR as a homodimer in the non-ligated state or as a heterodimer consisting of VDR and RXR upon binding of vitamin D [162]. With regard to its mechanism of action, it might be justified to call it a hormone rather than a vitamin. A tremendous amount of research regarding the involvement of vitamin D in a wide spectrum of diseases, predominantly autoimmune mediated diseases but also cancer has been performed within the last years. This is especially interesting with regard to the geographical distribution of certain diseases such as multiple sclerosis and their association with the level of sun exposure and vitamin D trough levels in individuals living at different latitudes. Examples for the immunomodulatory mechanisms of vitamin D are given by a study which showed that calcitriol inhibited Th9 differentiation *via* the aryl hydrocarbon receptor (AhR) through an IL-10 dependent mechanism in human T cells [163]. Other studies showed

that calcitriol increases the expression of the purine ectonucleotidases CD39 and CD73 in human peripheral CD4⁺ T cells, which are supposed to downregulate inflammation by regulating extracellular levels of adenosine [164]. Another study performed in asthmatic individuals clearly showed that serum 1,25-dihydroxyvitamin D levels positively correlated with the number of CD4⁺CD25⁺Foxp3⁺CD127⁻ Treg cells in peripheral blood and that, in fact, steroid-resistant asthmatics have significantly lower numbers of such Tregs [165]. That 1,25-dihydroxyvitamin D also increases the immunomodulatory activity of pre-existing tTreg to suppress Th2-skewed immune responses and have, indeed, the capacity to regulate airway hyperresponsiveness [166]. Moreover, deficiency of dietary vitamin D *in utero* or perinatally was shown to shift the Th balance towards the Th2 phenotype, which was accompanied by reduced IL-10 secretion levels in mice challenged with house dust mite extracts [167]. However, when analyzed in T cell clones, some studies reached the conclusion, that vitamin D rather promoted the expansion of Th2 clones and their secretion of IL-4 and IL-13, especially under when combined with IL-4 [168]. In humans, vitamin D reduced the expansion of Th17 cells in asthmatic children and reduced the number of IL-17-secreting T cells in adults with atopic conditions [169, 170].

Notably, a recently closed randomized, double-blind, placebo-controlled trial investigating the role of oral vitamin D supplementation in individuals with asthma and vitamin D deficiency was unable to show a benefit with regard to first treatment failure and exacerbation rates [171]. In addition, also a meta-analysis of clinical trials investigating the role of oral vitamin D supplementation in asthmatic individuals could not confirm any improvement of disease-related symptoms or objective parameters such as lung function and exacerbation rates [172]. A second meta-analysis concluded that some evidence for a reduction of asthma exacerbations by vitamin D supplementation is provided but the role of supplementation for other asthma related outcomes is currently inconclusive [173].

5. Principle mode of action and effects on allergen-specific T lymphocytes of novel drugs targeting specific T cell functions

5.1. Biologics blocking the action of Th2 cytokines

The Th2 cytokines IL-4, IL-5 and IL-13 represent hallmarks of allergic immune reactions and play an important role in allergic asthma and atopic dermatitis [174]. They not only attract and activate a multitude of immediate effector cells but also represent important differentiation factors for T cells. In fact, the absence of IL-4 inhibits differentiation of Th0 cells towards Th2 cells [175, 176]. Notably, the IL-4R and the IL-13R share the same high-affinity α -chain. Very recent clinical studies in atopic dermatitis patients have shown that targeting of the IL-4R α -chain with the monoclonal antibody dupilumab improves pruritus, symptoms of anxiety and depression as well as quality of life [177]. While separately targeting of IL-4 or IL-13 did not improve allergic asthma, beneficial effects of anti-IL-4R α treatment were clearly evident in such patients [178, 179]. Whether IL-4R α blockade also impacted directly on Th cells expressing receptors for IL-4 and IL-13 or whether the beneficial effects observed are the results of targeting innate effector cells remains to be shown in future studies. The combination of Th2 cytokine receptor targeting drugs with other substance classes could represent very elegant, novel treatment options to reduce toxicity and increase treatment efficiency for difficult-to-treat patient collectives.

5.2. Kinase inhibitors and other targeted drugs

As anticipated for JNK in the previous section, another strategy to reduce GC-mediated side effects would be to lower the dose or even completely replace GC treatment by other substances, *e.g.* targeted drugs ideally exerting selected immunosuppressive effects on Th2 cells. Examples for clinically tested targeted drugs for the treatment of atopic conditions are the Syk-kinase inhibitor R112 for allergic rhinitis asthmatics [180, 181], and masitinib, a c-kit/PDGF-receptor tyrosine kinase inhibitor for corticosteroid-dependent asthmatics [180,

181]. The primary intention for the local application of a Syk-targeting drug was certainly the involvement of Syk in FcεRI-mediated signaling in mast cells [182, 183], however, Syk is also well known for its role in regulating TCR-dependent signaling where it promotes phosphorylation of ITAMs within the TCR complex [184]. Similarly, masitinib was clinically evaluated because of its prominent role in inhibiting the c-Kit/PDGF signaling pathway, which regulates the function of mast cells and bronchial structural cells. Although, masitinib is regarded to be a highly-specific c-Kit/PDGF-inhibitor [185], some reports also suggested blocking activity for the T cell specific TK Lck [186].

Inhibition of phosphoinositide kinase-3δ showed promising results in *in vitro* experiments performed with T cells from asthmatic patients with regard to inhibition of T cell activation and cytokine secretion and would be an interesting target since, apart from T cells, this kinase is also involved in B cell and mast cell activation [187, 188]. Furthermore, the SHIP1 activator AQX-1125, which is supposed to enhance the endogenous inhibition of the phosphoinositide-3-kinase pathway, was well tolerated and led to significantly reduced late responses to allergen challenge in a randomized, double-blind, placebo-controlled, two-way crossover study [189]. Another target that has been shown to be reasonable in mouse models of asthma and in human cells from asthmatics *in vitro* is the Janus kinase 1/3 (JAK 1/3), which is involved in signaling important for Th2 immune responses [187, 190]. Furthermore, the tyrosine kinase inhibitor Dasatinib, which is known to efficiently inhibit SFK and Abl family kinases and which represents an important alternative for the effective treatment of Imatinib-resistant or -intolerant CML-cases [191], was evaluated in a mouse model of asthma, which showed promising results with regard to lung inflammation and remodeling [192] and also had beneficial effects in patients with contact dermatitis [193]. Also these findings are not entirely surprising since *Schade et al.* have clearly shown that Dasatinib is able to block the earliest events in TCR signaling even at single-digit nanomolar concentrations [194]. Besides its clear T cell modulatory capacity, Dasatinib has also been shown to significantly

impact on mast cell function by inhibiting FcεRI-dependent mast cell degranulation [195]. Also, evaluated in *in vivo* experiments was the selective inhibition of IκB kinase (IKK) β by IMD-0354, which prevented mice from ovalbumin-induced airway inflammation through inhibition of activation and proliferation of CD4⁺ T cells in mediastinal lymph nodes [196]. That the IκB kinase 2 inhibitor IMD-0354 also inhibits human T cells has been shown in studies targeting adult T cell leukemia specimen, which are driven into growth arrest or even apoptosis by the inhibitor [197].

5.3. SiRNA and DNAzymes

Small interfering RNAs represent double-stranded RNA molecules, which have the ability to interfere with gene expression by interacting with complementary mRNA sequences of the targeted gene [198]. The use of small interfering (si) RNA to inhibit the lymphocyte-specific protein tyrosine kinase Lck in a mouse model of asthma reduced T cell specific mRNA expression of Lck and resulted in reduced levels of IL-4 and IgE in broncho-alveolar lavage fluids when these mice were pretreated with siRNA [199].

Another class of catalytically active, nucleotide-based molecules are represented by DNA enzymes (DNAzymes) [200]. A DNA enzyme has first been described by *Breaker and Joyce* in 1994 and several representatives have been characterized by *in vitro* selection procedures from large sequence pools until today [200, 201]. Along those lines, a highly interesting development represents the DNAzyme SB010, whose active product hgd40 is composed of a single strand DNA of 34bp length that forms GATA-3 mRNA-specific domains and a catalytically active center that allows for specific binding to and cleavage of GATA-3-specific mRNA, which represent the signature transcription factor of the majority of allergen-specific Th2 cells [3, 202, 203]. Applied locally as an inhalant it was tested in a randomized placebo controlled clinical trial showing promising results with significant reduction of early and late

asthmatic responses [3]. The future will show, whether systemic application of targeted versions of allergen-specific DNAzymes can be realized.

Conflict of interest disclosure

Winfried F. Pickl: In the last five years consultant and lecture activity with and without relation to individual products: Novartis; Shareholder Biomay AG, Vienna, Austria.

Acknowledgements

This work was supported by the Austrian Science Fund (FWF) project SFB F4609 and DK W1248.

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Legend to Figure

Fig. 1. Overview on targets typically modulated by anti-allergic drugs.

Figure shows a schematic cell with relevant transmembrane receptors and ion channels inserted into the plasma membrane. The cytosol contains different signaling and metabolic pathways and a nucleus containing DNA and relevant transcription factors. The different anti-allergic and immunosuppressive drugs and novel experimental substances (red font) and their targeted structures (red arrows) are depicted. Messenger RNA targeted by a DNAzyme is shown in blue.

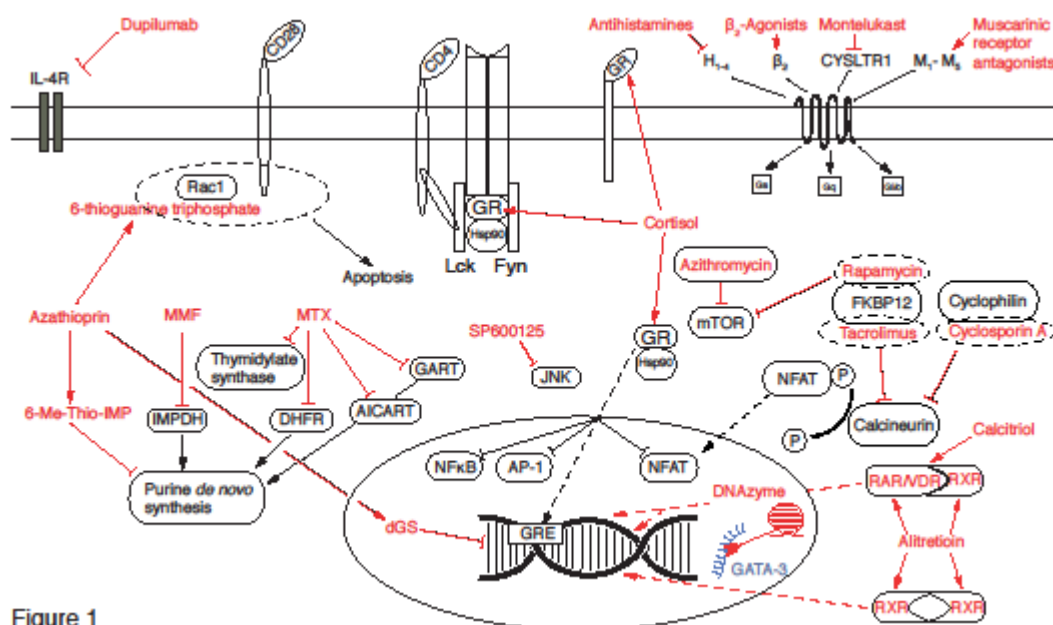


Figure 1