The effect of IL-2, in combination with 1,25(OH)2D3,Followed by Zinc as Inhibitory and Potential Immunomodulatory in Cancer and Autoimmunity Treatment and Prevention

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Abstract: The disrupted balance between regulatory and an effector T cell (Tregs and Teffs, respectively) is a characteristic of autoimmune diseases, and is dependent on homeostatic cytokines, including IL-2. All preclinical and clinical studies discussed emphasize the potential therapeutic benefit of low-dose IL-2 therapy of autoimmune disorders.

Recent studies of the functional, biophysical and structural characteristics of IL-2 have led to the generation of IL-2 formulations, including IL-2/mAb complexes and IL-2 variants (muteins) that selectively enhance IL-2's immune stimulatory versus inhibitory properties.

IL-2 is the major growth factor optimizing Tcell responses as signaling through its high affinity IL-2 receptor (consisting of the a, b and common c chains) and the Jak3- Stat5 axis is essential for the survival, proliferation and differentiation of antigen-activated T cells.

Recent epidemiologic studies have observed relationships between low 1,25(OH)2D3 levels and multiple disease states. Low 1,25(OH)2D3 levels are associated with increased overall and cardiovascular mortality, cancer incidence and mortality, and autoimmune diseases such as multiple sclerosis.

Recent reports have supported a role for 1,25(OH)2D3 in mediating normal function of both the innate and adaptive immune systems. Crucially, these effects appear to be mediated via localized autocrine or paracrine synthesis of 1,25(OH)2D3 from precursor 25- hydroxyvitamin D3 (25OHD3), the main circulating metabolite of 1,25(OH)2D3.

In this article, I discuss the Genenomic evidence for a role of IL-2 in autoimmunity, IL-2 Immunotherapy in an Animal Model of Autoimmunity and the effects of IL-2 in cancer as well as 1, 25 (OH)2D3, molecular mechanisms and immune modulation and1, 25 (OH)2D3, and Cancer.Moreover, IL-2, in combination with 1,25(OH)2D3,followed by Zinc, Inhibitory and Immunomodulatory, Potential in Cancer and Autoimmunity.

Key Word:IL-2, 1, 25 (OH)2D3, Zinc, Inhibitory, Immunomodulatory, Animal Model, Cancer, and Autoimmunity

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1. Introduction

Interleukin-2 (IL-2) is produced by activated T cells. IL-2 was first described as a T cell growth factor. It is now known to stimulate growth and differentiation of B cells, natural killer (NK) cells, lymphocyte activated killer (LAK) cells, monocytes/macrophages and oligodendrocytes. Mouse IL-2 is species specific and is inactive on human cells. The biological activity of IL-2 is mediated by the binding to a cell surface receptor. The functional high affinity receptor of IL-2 is composed of three distinct polypeptide chains, the IL-2 receptor α, β and γ subunits. The intermediate-affinity IL-2 receptor complex, which lacks the a subunit, but contains both the β and γ subunits, is also capable of transducing the IL-2 signal. The γ chain of the IL-2 receptor complex has been shown to be a subunit of the receptor complexes of IL-4, IL-7, IL-9 and IL-15. Biological activity of IL-2 is mediated by JAK1 and JAK3-induced activation of STAT3 and STAT5.

Vitamin D receptor (VDR) and shuttles between the cytoplasm and the nucleus (nuclear VDR, nVDR). Cellular action only follows after binding of 1,25-(OH)2D by the nVDR in the target cell. The gene encoding the VDR is located on chromosome 12q14 and has several common allelic variants
(1).

The nVDR is a member of the nuclear steroid, retinoid, and thyroid hormone receptor superfamily, acts as a ligand activated transcription regulator, and 1,25-(OH)2D is a ligand. The activated VDR dimerizes with another nuclear receptor, the retinoic acid receptor (RXR). The heterodimer RXR/VDR/1,25-(OH)2D binds to a vitamin D responsive element (VDRE), a specific sequence of DNA, in the promoterregion of target genes, regulated by 1,25-(OH)2D. Upon binding to the VDRE, the heterodimer RXR/VDR/1,25- (OH)2D activates or suppresses gene transcription, whereby synthesis of proteins is induced or repressed. 1,25-(OH)2D thus exerts biological actions through VDR-mediated geneexpression dependent on the target cell(2).

Zinc is a biologically essential trace element; critical for cell growth, development and differentiation (3). It is required for DNA synthesis, RNA transcription, cell division, and cell activation(4), and is an essential structural component of many proteins, including signaling enzymes and transcription factors. Zinc is required for the activity of more than 300 enzymes,

interacting with zinc-binding domains such as zinc fingers, RING fingers, and LIM domains (5),(6),(7). The RING finger domain is a zinc finger which contains a Cys3HisCys4 amino acid motif, binding two zincs, contains from 40 to 60 amino acids. RING is an acronym specifying Really Interesting New Gene. LIM domains are structural domains, composed of two zinc finger domains, separated by a two-amino acid residue hydrophobic linker. They were named following their discovery in the proteins Lin11, Isl-1 and Mec-3. LIM-domain proteins play roles in cytoskeletal organization, organ development and oncogenesis. More than 2000 transcription factors have structural requirements for zinc to bind DNA, thereby revealing a critical role for zinc in gene expression.

2. Biology and immunologyInterleukin-2

Interleukin-2 (IL-2), as well as other members of the IL-2-related family of T cell growth factors (e.g., IL-4, IL-7, IL-9, IL-15, and IL-21), utilize a common receptor signaling system that results in the activation and expansion of CD4+ and CD8+ T cells. The biological effects of IL-2, a 15.5 kDa variably glycosylated protein comprised of four antiparallel α-helices, are mediated by the IL-2 receptor, a trimeric complex composed of βCD25, γ(CD122) and γ (CD132) chains. The β and γchain are involved in signaling, while the ligandspecific chain is only involved in cytokine binding. These subunits form a high (βγ), intermediate (βγ) or low affinity receptor (β) depending on which of the chains are in the cell surfacecomplex(8),(9). Although the β and γ chains are expressed on T cells, B cells and NK cells (10), the γ chain is inducible and is expressed only by T cells but is present on several phenotypically and functionally distinct classes of T lymphocytes. The predominant cellular source of IL-2 is the CD4 T cell, specifically the Th1 subset, and its major physiologic role of IL-2 is to promote the activation and proliferation of T and NK cells in an autocrine and paracrine manner (11).In contrast to T cells, NK cells express the intermediate affinity IL-2 receptor (no subunit). Exposure of NK cells to IL-2 results in proliferation, enhanced cytolytic activity and secretion of other cytokines. B cells also express intermediate affinity IL-2 receptors and can secrete IL-2 in cooperation with other cytokines, resulting in B cell proliferation and differentiation (10). IL-2 also plays a major role in suppressing T cell responses. A subpopulation of CD4+ T cells, characterized by high levels of CD25 and the forkhead/winged helix transcription factor FoxP3, function to suppress self-reactive T-cells. These regulatory T cells (Tregs) maintain tolerance and prevent autoimmunity after activation of effector T-cell responses (12). In murine models, depletion of CD4+FoxP3+ Tregs enhances tumor rejection and improves therapeutic responses to cancer vaccines by promoting the function of CD8+ cytotoxic T lymphocytes (13). The mechanisms by which Tregs inhibit the function of CD8+ CTLs are incompletely understood. However, recent *in vivo* studies show competition for IL-2 is a critical pathway by which Tregs limit CD8+ T cell expansion and effector differentiation (14). Furthermore, the loss of IL-2 signaling, as demonstrated in mice with a targeted deletion of IL-2 or the IL-2 receptor, leads to a generalized inflammatory syndrome and an often fatal autoimmune colitis (13),(15),(16),(17),providing additional evidence of the role of IL-2 not only as an activator of immune responses but also as a key mediator of immune tolerance. This relatively recent insight into IL-2 as a regulatory cytokine, rather than a purely stimulatory T cell growth factor, suggests that the use of IL-2 in the clinical setting needs to be re-evaluated. An important area of further investigation will be a more

careful analysis of the dosing, schedule and kinetics of IL-2 administration on specific T cell subsets.

3. Genenomic evidence for a role of IL-2 in autoimmunity

The IL-2-IL-2R pathway plays an essential role in the development of T1 D and other autoimmune disorders in humans and mice. IL-2 is well-described to promote activated T cell proliferation, survival and differentiation (18). However, mice deficient for IL-2, IL-2Ra (CD25) or IL-2Rb (CD122) die prematurely from a severe, multiorgan, autoimmune and lymphoproliferative disorder (19). Similarly, rare genetic disorders due to mutations of the il2, cd25 or stat5a/b genes lead to autoimmune syndromes (20),(21),(22), emphasizing the importance of IL-2 in the maintenance of self-tolerance (19).

4. Role of IL-2 in stabilizing Foxp3+ Treg cells

Several findings suggest that IL-2/IL-2R signaling is necessary for the peripheral maintenance and fitness of Treg cells. In Fontenot et al., the analysis of Foxp3-GFP reporter knock-in mice genetically deficient for IL-2 or IL-2R (CD25) revealed that IL-2 signaling is not required for the induction of Foxp3 expression in thymocytes. These findings were further confirmed by demonstrating that Treg cell development is independent of IL-2, while this cytokine is essential for survival of Treg cells (23). Moreover, although IL-2-/- or IL-2R-/- mice display reduced numbers of Treg cells in vivo, their suppressive function in vitro remains unaffected (24). Nonetheless, gene expression analysis showed that IL-2 signaling was required for the maintenance of the expression of the genes involved in the regulation of cell growth and metabolism (25). Hence, IL-2 has a critical role in the homeostasis and competitive fitness of Treg cells (26). Interestingly, the adoptive transfer of WT Treg cells either in IL-2-/- or IL-2R-/- mice can only prevent autoimmunity in IL-2R-/-, and not IL-2-/-, mice (27). These results indicate that the lack of Treg cells in IL-2-/- and IL-2R-/- mice contributes to the autoimmune phenotype and that IL-2 maintains self-tolerance by increasing the number of Treg cells present in the peripheral organs (28).

5. The effects of IL-2 in cancer

Based on the preclinical model experiments, the first clinical trials using local IL-2 administration were performed (29), used the transurethral administration of IL-2 into the immediate vicinity of urinary bladder carcinomas. Repeated injections of IL-2 under cystoscopic control resulted in a complete regression of the tumour in three out of ten patients and in partial regression in another three patients. The regressions were estimated by the biopsy and with the help of cystoscopic examination; complete regressions lasted over the whole observation period, 2, 4, and 7 months. The first pioneer experiments performed with IL-2 in humans were repeated with other tumour types (30), injected IL-2 into the pleural exudate of patients suffering from bronchogenic carcinomas; in nine of the 11 patients treated in this way, exudates were resorbed between the 4th and the 10th day after starting the therapy and tumour cells disappeared in the pleural fluid (31), observed in six out of 23 patients with recurrent malignant gliomas, treated by repeated injections of IL-2 + LAK cells directly into cavities of tumours, regressions of the

gliomas. Those were patients non-responding to conventional radiochemotherapy and surgical therapy; in three out of the six successfully treated patients the injection of IL-2 induced a remission lasting more than 6 months. A group of Forni(32),(33), used IL-2 for the therapy of patients with recurrent spino-cellular carcinomas of the head and neck. Interleukin-2 was injected into the region of lymphatic vessels at the site of the insertion of the sternocleidomastoid muscle to the process susmastoideus; IL-2 was administered repeatedly, ipsilaterally to the location of the tumour process. Tumours of six out of ten patients responded to the therapy with IL-2; of the six tumours, three responded by complete regression and those of another three patients by partial regression. The positive response to the therapy was observed only in patients without a radical excision of cervical lymphatic nodes. The remission was present throughout the time of the observation, i.e. 5 to 8 months (32), However, when these successful head and neck cancer treatment results were re-evaluated after a longer period, and repeated by other groups, the results were substantially less optimistic (34),(35),(36),(37). In addition to the data reported by (29),(38),described complete, histologically confirmed remission lasting more than 6 months in one out of five patients with urinary bladder carcinoma after continuous IL-2 perfusion of the bladder performed for 5 days. The first experiments exemplified in Table 5 were extended and repeated by other groups and their results were not substantially different (39),(40),(41),(42). A novel way of regional IL-2 administration was an inhalation therapy, proposed by (43). High-dose IL-2 inhalation therapy with very low toxicity and low level of adverse effects has been used in more than 200 patients with metastatic renal cell carcinomas, melanomas, breast and ovarian carcinomas (43),(44),(45). When the results obtained during 6 years of inhalatory IL-2 therapy in 116 metastatic renal cell carcinoma patients were evaluated, it was concluded that the progressive pulmonary metastases responded dramatically in 15% of patients for a median of 15.5 months and were stabilized in 55% of patients for a median of 6.6 months (46).

6. Physiological role for Vitamin D in Immune Regulation

Considering the short half-life time of bioactive 1,25(OH)2D3 (4–6 h) and the supra physiological concentrations of 1,25(OH)2D3 required to modulate the behavior of immune cells, it is rather unlikely that its immunomodulatory actions would depend on systemic levels of the hormone. In this context, the discovery of expression of vitamin D metabolizing enzymes in various target cells of 1,25(OH)2D3, comprising the majority of immune cells, caused a major breakthrough in understanding the non-classical actions of 1,25(OH)2D3. Various immune cells, including macrophages, DCs, and even B-lymphocytes and T-lymphocytes were found to express CYP27B1, while DCs also express CYP2R1 (47), (48),(49),(50). The theory of local vitamin D metabolism within the immune system is further supported by the demonstration that all these cell types are capable to convert 25(OH)D3 into bioactive 1,25(OH)2D3, allowing them to respond not only to the active vitamin D metabolite, but also to its precursors (51),(47), (48). Importantly,regulation of CYP27B1 in immune cells is remarkably different from the renal counterpart, since its expression is controlled by immune signals. For example, CYP27B1 expression by monocytes/macrophages is strongly upregulated by IFN-g, the TLR4- ligand LPS, ligands triggering the TLR2/1-complex such as the 19 kDa lipoprotein of Mycobacterium tuberculosis, and viral infections(51), (50),(52),(53). In DCs, CYP27B1 levels increase during maturation of these cells,while in Tlymphocytes and B-

lymphocytes, expression of this enzyme is dramatically enhanced upon activation of the cells (54),(47),(55). In further contrast with renal CYP27B1, expression of this enzyme in macrophages and DCs is not suppressed by 1,25(OH)2D3 itself (50), offering an explanation for the massive local production of 1,25(OH)2D3 by disease-associated macrophages in patients with granulomatous diseases. However, the ability of 1,25(OH)2D3 to trigger CYP24 in immune cells is likely to serve as a negative feedback loop. Importantly, the susceptibility of monocytes and macrophages to this 1,25(OH)2D3-mediated induction of CYP24 depends on the differentiation/maturation stage of the cells, as undifferentiated monocytes are highly sensitive to 1,25(OH)2D3-mediated CYP24 induction, whereas differentiated/activated macrophages are not (56). Together, local processing of vitamin D precursors into the active ligand represents an important mechanism by which immune cells can reach the supraphysiological levels of 1,25(OH)2D3 needed to shape immune responses, without affecting systemic levels of this hormone. Therefore, the presence of VDRs and vitamin D metabolizing enzymes and their regulation by immune signals provide strong evidence for an autocrine and/or paracrine role for 1,25(OH)2D3 in normal immune physiology.

7. Effects of Vitamin D on the Immune System

Based on the ectopic production of vitamin D in cells of the immune system and the presence of VDR in tissues that are not related with bone physiology, the immunoregulatory properties of vitamin D have been better characterized(57). Epidemiological studies have shown that deficiency of this vitamin could be associated with an increased risk of colon and prostate cancer, cardiovascular disease, and infections(58),(59),(57),(60). Several mechanisms have been proposed to explain the role of vitamin D in the physiology of the immune system, as can be seen in Table 1. Among the main functions of vitamin D in the immune system, we could mention: regulation of the differentiation and activation of CD4 lymphocytes; (61),(62),increase in the number and function of regulatory T cells (Treg); (62), in vitro inhibition of the differentiation of monocytes in dendritic cells; (63),(62), reduction in the production of cytokines, interferon-g, IL-2, and TNF-α by Th1 cells, and stimulation of the function of Th2 helper cells; (61),(63),(62), (57). inhibition of the production of IL-17 by Th1 cells; (64), and in vivo and in vitro stimulation of NK T cells(65).

8. Vitamin D molecular mechanisms and immune modulation

The effects of 1,25(OH)2D are mediated by it binding to the vitamin D receptor (VDR). VDR is a nuclear receptor and once it binds its ligand, VDR dimerizes with an isoform of the retinoid X receptor. These VDR-RXR heterodimers bind to vitamin D response elements present on target genes (66),(67),(68). In addition to transcriptional activation, the heterodimers can displace the nuclear factors of activated T cells resulting in repression of cytokine related genes(69). 1,25(OH)2D suppresses Th-1 cell proliferation leading to lowered production of interferon gamma and interleukin-2(70),(71). Lower levels of circulating cytokines leads to less antigen presentation by dendritic cells, in addition to less T lymphocyte recruitment and proliferation (71). Expression of Th-2 associated cytokines, including interleukin-4 are increased by 1,25(OH) (72). Overall, vitamin D polarizes the adaptive immune system away from Th-1 and toward Th-2 responses. Vitamin D also plays a role in innate immune response modulation. The toll-like receptors (TLRs) in macrophages, polymorphonuclear cells, monocytes, and epithelial cells are central to the innate immune response (73),(74). TLRs

recognize pathogen associated molecular patterns associated with infectious agents(74). For example, TLR2 recognizes the lipopolysaccharides of bacteria. TLRs have also been shown to recognize viral proteins and nucleic acids (75). Upon recognition, activated TLRs release cytokines that induce expression of antimicrobial peptides and reactive oxygen species. Several TLRs both affect and are affected by VDR stimulation. Expression of CD-14, the co-receptor for TLR4, is induced by 1,25(OH)2D in monocytes and epidermal keratinocytes (76). Stimulation of TLR2 in macrophages by anti-microbial peptides leads to increased local expression of CYP27B1, resulting in the conversion of vitamin D to its active form. Some anti-microbial peptides associated with TLRs have demonstrated antiviral effects, and their expression is affected by vitamin D levels(77). Human beta defensin 2 is modestly up-regulated by 1,25(OH)2D and may contribute to anti-viral effects as a chemoattractant for neutrophils and monocytes (73),(78). Conversely, in monocytes activation by 1,25(OH)2D alone is insufficient for induction of gene expression (79). Human cathelicidin is an antimicrobial peptide induced by TLR1/2 activation. Cathelicidin is strongly up-regulated by 1,25(OH)2D due to the its VDR response element (79),(80),(81).Cathelicidins are a family of proteins with a C-terminal cationic anti-microbial domain activated by cleavage from the N-terminal cathelin domain(81). In humans, the active antimicrobial cathelicidin peptide LL-37 is cleaved from the propeptide, hCAP18.(82). Although the majority of cathelicidin is stored in neutrophil granules for release at sites of infection, several other types of immune cells including monocytes, NK cells, and B cells express hCAP18 (83). It is secreted into the blood and by the epithelia of the conjuctiva, cornea, respiratory tract, digestive tract, epithelial tract, intestines, urinary tract, and skin (84),(85),(86),(87). At the cellular level, expression of CYP27B1 in macrophages and keratinocytes induces cathelicidin expression.If there is no 25(OH)D, VDR, or CYP27B1 present, the ability of these cell types to induce cathelicidin is significantly impaired.
In addition to anti-bacterial effects including membrane disruption (86),(87),(88), cathelicidininthe peptide form LL-37, has demonstrated anti-viral effects including inhibition of herpes simplex virus type one (HSV-1), vaccinia virus replication, retroviral replication, and replication of some adenovirus serotypes at certain peptide concentrations (85),(89),(90),(91).

9. Vitamin D and Cancer

Although carcinogenesis can occur relatively quickly, most cancers develop over decades making it difficult to perform reliable human intervention studies on the association between vitamin D and cancer risk. However, there is evidence that enhanced sunlight exposure is associated with lower prostate, breast and colon cancer death rates, while the historical geographical distribution of rickets parallels that for these cancer deaths (92). The strongest epidemiological evidence supporting a protective role for vitamin D in colon cancer is from prospective studies. Inverse associations for vitamin D intake and colon or colorectal cancer with relative risks ranging from 0·33 to 0·74 have been reported (93),(92). Moreover, a nested case–control study based on serum drawn from a cohort of 25 620 individuals reported that concentrations of 25(OH)D in the range of 65–100 nmol/l were associated with large reductions in the incidence of colorectal cancer compared with lower 25(OH)D levels (94). A reduced risk of breast cancer has been observed in theNHANES I epidemiological follow-up study with several measures of sunlight exposure and dietary vitamin D intake, with relative risks ranging from 0·67 to 0·85. The risk reductions were highest for women who lived in US regions of high solar radiation and no reduction was founds for women who lived in regions of low solar

radiation (95). Data are in line with experimental results suggesting that high amounts of vitamin D and dietary Ca decrease susceptibility to chemically induced mammary Neoplasia(96). Another important observation is that in the USA the occurrence of prostate cancer and MS have similar geographical distributions (97). The hypothesis of a vitamin D dependency on prostate cancer has recently been confirmed by a large nested case–control study (98). In a 13-year follow-up study of about19 000 middle-aged Finnish men, prostate cancer risk
was highest among the group of younger men (40–51 years) with low serum 25(OH)D levels. Approximately one half of the serum samples had 25(OH)D levels below 50 nmol/l. Low serum 25(OH)D levels, however, appeared not to increase the risk of prostate cancer in older men (.51 years). Data suggest that vitamin D has a protective role against prostate cancer only before the andropause, when serum androgen concentrations are higher. The lowest 25(OH)D concentrations in the younger men were associated with more aggressive prostate cancer (98). Vitamin D is anti-proliferative and promotes cellular maturation, induces differentiation and apoptosis in many different cell lines including malignant cells (99),(92). Vitamin D receptors have been found in the mammary gland, in the colon and in the prostate (Table 1). Moreover, it is now recognized that colon, breast, and prostate cells also express the 1ahydroxylase to form calcitriol from circulating 25(OH)D (100).It seems clear that vitamin D must be viewed as an important cellular anti-tumour substance.

10. Zinc and the Immune Response

Zinc deficiency affects multiple aspects of innate and adaptive immunity, the consequences of which in humans include thymic atrophy, altered thymic hormones, lymphopenia, and compromised cellular-and antibody-mediated responses that result in increased rates and duration of infection. Zinc deficiency also plays a role in the immunosenescence of the elderly (101). Changes in gene expression for cytokines, DNA repair enzymes, zinc transporters, and signaling molecules during zinc deficiency suggest that cells of the immune system are adapting to the stress of suboptimal zinc (102). Furthermore, oral zinc supplementation improves immunity and efficiently down-regulates chronic inflammatory responses. These general findings suggest that zinc is critical for normal immune cell function, whereby zinc depletion causes immune cell dysfunction, and zinc supplementation can either restore function in the setting of dysfunction or improve normal immune cell function (103).

11. Conclusion

Some studies have suggested that IL-2 used in combination with other agents might be more effective than IL-2 alone in the treatment of leukemia. For example, IL-2 has been combined with histamine in the treatment of AML with surprisingly good results. Another strategy might be to combine IL-2 with interferon alpha since the latter may up-regulate the expression of MHC class 2 molecules leading to better tumor recognition.

Given the strong link between IL-2 and autoimmunity, it seems appealing to consider the use of IL-2 as a therapeutic tool for T1 D. However, this might prove quite challenging, as IL-2 is first and foremost a T cell growth factor, and as such, has strong proliferative effects on all T cells,

including pathogenic CD4+ and CD8+ Teff cells. For the past decade, IL-2 has been used in the treatment of several diseases where the immune system necessitates strengthening of the activated T cell pool.

Vitamin D, in addition to its crucial role in bone metabolism, has been associated with multiple autoimmune diseases in several epidemiological studies. Due to its unique capability to bind to VDR and serve as a transcriptional factor, vitamin D can regulate gene expression and further exert its immunomodulatory effects on immune cells.

Furthermore, accumulating evidence suggest that VDR polymorphisms and serum vitamin D status are both closely associated with disease risk of MS, T1DM, and SLE. Therefore, impaired vitamin D signaling and/or inadequate vitamin D intake caused by genetic predisposition (e.g. VDR polymorphisms) and/or environmental factors (e.g. insufficient sunlight exposure in high-latitude regions or during the cold season) may contribute to the onset and progression of autoimmunity.

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