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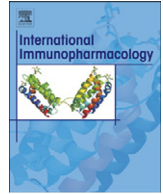

Induction of pro-inflammatory cytokine production in thymocytes by the immune response modifiers Imiquimod and Gardiquimod™

Andreas Weber [1], Corinna Zimmermann [1], Anne K. Mausberg, Bernd C. Kieseier, Hans Peter Hartung, Harald H. Hofstetter *

Department of Neurology, Heinrich Heine University, Düsseldorf, Germany

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ABSTRACT

An emerging role is postulated for IL-17-producing thymocytes, which in their majority consist of IL-17-producing CD4$^+$ cells. For these, a specific role in the immediate defense against infectious pathogens is suggested, independent from the development of an adaptive immune response in the immune periphery. Immune response modifiers, like the TLR7 ligands Imiquimod and Gardiquimod™ are effective pharmacological therapeutics applied topically against dermal tumors and virus infections and have been demonstrated to activate immune cells. In this study, we investigated the effect of Imiquimod and Gardiquimod™ on murine thymocyte cytokine production with a particular focus on IL-17. We find that both substances dose-dependently are able to trigger IFN-γ and IL-6 production, but no IL-17 production. Moreover, a strong co-stimulating effect is detected on α-CD3-induced IFN-γ, IL-6 and IL-17 production. We conclude that Imiquimod and Gardiquimod™ are not only modifiers of the adaptive immune response, but might also have additional therapeutic potential by modifying the immune activity in the thymus.

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1. Introduction

Interleukin-(IL-) 17-producing T cells play a critical role in the immune response against infectious pathogens and tumors as well as in the pathogenesis of autoimmune disorders [1]. In experimental studies, traditionally induced IL-17-producing T cells have been investigated [2,3]. In addition, a population of IL-17-producing T cells exists in the thymus of naïve wild-type mice, which predominantly consists of CD4 positive cells [4,5] and therefore have been termed naturally occurring IL-17-producing T cells [6,7]. As a main task of these cells, immediate reactivity against microbes is assumed, thereby bridging adaptive and innate immune response [7]. They differ in their activation requirements from induced T cells [5,8] and need thymic dendritic cells to develop [9]. Toll-like receptors (TLR) play an important role in the innate immune response as well as in the polarization of an adaptive immune response and can both directly and indirectly modulate T-cell function. Some TLR ligands present a promising class of immunotherapeutic or vaccine adjuvants with the potential to produce an effective antitumor immune response [10]. TLR7 is localized to intracellular membranes by its trans-membrane domain and contains targeting sequences for the induction of immune responses against viral infections [11]. Stimulation of TLR7 via TLR7 ligands leads to an activation of antigen-presenting cells, including dendritic cells [12]. Examples for pharmacological TLR7 ligands are Imiquimod and Gardiquimod™, which are also termed immune response modifiers. Imiquimod is an imidazoquinoline amine analog to guanosine with potent indirect antiviral activity, which is already in use with clinical efficacy and safety as topical treatment for external genital warts caused by human papillomavirus infection [13,14] as well as other cutaneous tumors and herpes simplex disease [15]. Moreover, topical application in an experimental setting can also yield a therapeutic benefit for tumors in other anatomic localizations, e. g. intracranial or breast carcinoma [16]. Gardiquimod™ is a newly developed compound manufactured by InvivoGen (San Diego, CA, USA). While Gardiquimod™ activates both TLR7 and TLR8, Imiquimod mainly activates TLR7 and only marginally TLR8 [13,17]. Systemic application of immune response modifiers for virus infections like HIV and HCV, e. g. with masked oral prodrugs of TLR7 agonists is discussed [18], but so far no clinically approved treatment option is available. Furthermore, recent studies indicated that an effective drug to treat benign skin abnormalities containing Imiquimod triggers IL-17 production, whereby γδ-T-cells and RORγt$^+$ innate lymphocytes are the main producing skin cell-subpopulations [19]. Since TLR7 stimulation has been shown to strongly activate thymocytes [20] and thymic dendritic cells are essential for the development of IL-17-producing thymocytes [9], we here investigated the influence of the immune response modifiers Imiquimod and Gardiquimod™ on the pro-inflammatory cytokine production in the thymus with a particular focus on IL-17. Using an ex-vivo model which we have established in previous experimental studies [4,8] we investigated both the effect of Imiquimod and Gardiquimod™ alone as well as in combination with

* Corresponding author at: Department of Neurology, Medical Faculty, Heinrich Heine University, Moorenstr. 5, 40225 Düsseldorf, Germany.
E-mail address: harald.hofstetter@uni-duesseldorf.de (H.H. Hofstetter).

[1] These authors contributed equally to this work.

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anti(α)-CD3 stimulation (thereby additionally activating thymocytes via the T cell receptor complex) in naïve wild-type C57.BL/6 mice.

2. Material and methods

2.1. Animals

Wild-type female C57.BL/6 mice at an age of 6–8 weeks were purchased from Centre d' Elevage R. Janvier (CERJ, Le Genest-St-Isle, France) and maintained at the local animal facilities. All animal experiments were fully approved by the local authorities for animal experimentation.

2.2. Cell preparation

After sacrificing the animals with isoflurane euthanasia, thymi were prepared. Subsequently, each thymus was squeezed using the back of a syringe plunger to obtain a single cell suspension. To sort out cell clusters, the obtained suspension was filtered through a 70 μm cell strainer (BD Biosciences, Heidelberg, Germany). The cells were counted by trypan blue exclusion and plated together with the respective stimulants at the cell numbers indicated in serum-free HL-1 medium (Lonza, Cologne, Germany).

2.3. Reagents

For α-CD3 stimulation, the clone 145-2C11 (BD Pharmingen, San Diego, USA) was used. Imiquimod and Gardiquimod™ were obtained from InvivoGen, San Diego, CA. Stimulation in cell culture was performed with 1 μg/ml for α-CD3 and in titration steps from 50–0.005 μg/ml for Imiquimod and 5–0.005 μg/ml for Gardiquimod™.

2.4. Cytokine measurement by ELISPOT and computer assisted ELISPOT image analysis

ELISPOT assays were essentially performed as described [4,8]. Briefly, $\text{MultiScreen}_{\text{HTS}}$ 96-well filtration plates (Millipore, Schwalbach Germany) were coated overnight with the capture antibodies in sterile PBS. Antibodies were ordered from BD Pharmingen, San Diego, CA. The coating antibodies for IL-6 (MPS-20F3) and IL-17 (TC11-18H10) were used at 2 μg/ml and for IFN-γ (R4-6A2) at 4 μg/ml. Plates were blocked for 1 h with sterile PBS/BSA 0.5% and washed 3 times with sterile PBS. Thymocytes (10^6 per well) were plated in HL-1 medium (BioWhittaker, Walkersville, MD) containing 1% glutamine and 1% penicillin/streptomycin in duplicate cultures each. Thereafter cells were stimulated with the different stimulation reagents and incubated for 24 h at 37 °C, 5% CO_2. Plates were washed 6 times with PBS before adding the detection antibodies (BD Pharmingen, San Diego, CA) overnight in PBS/BSA 0.5%. IFN-γ (XMG1.2) and IL-6 (MP5-32C11) were used at 2 μg/ml, IL-17 (TC11-8H4.1) at 0.5 μg/ml. After washing the plates for 4 times Streptavidin-AP (BD Pharmingen, San Diego, CA) in PBS/BSA 0.5% (1:500) was added for 2 h. After washing for another 4 times the plates were visualized using AP Conjugate Substrate Kit (BioRad Laboratories, München, Germany). Image analysis of ELISPOT assays was performed with the ImmunoSpot™ Analysis Software after scanning the plates with an ImmunoSpot™ Analyzer (Cellular Technologies, Cleveland, OH, USA).

2.5. FACS analysis of intracellular staining

Single cell suspensions of thymocytes were prepared and stimulated with 5–0.05 μg/ml Imiquimod and combined with α-CD3 for 24 h. Addition of BD GolgiStop™ for the last 12 h to the cells blocks their intracellular transport process. After blocking with BD Fc Block™ (clone 2.4G2) cells were stained with PE-labeled anti-CD4 (clone GK1.5) and APC-H7 labeled anti-CD8a (clone 53-6.7). Intracellular staining was then performed using BD Cytofix/Cytoperm™ plus Fixation/Permeabilization Kit Stop (BD Pharmingen) and PerCP-Cy™ 5.5 labeled anti-IL-17A (clone TC11-18H10). All antibodies were purchased from BD Biosciences, Heidelberg, Germany. FACS analysis was performed on a Millipore Guava EasyCyte™ 8 using GuavaSoft™ software version 2.2.2.

2.6. Statistical analysis

For statistical analysis, one-way ANOVA with Dunnett's two-tailed t test (Instat, GraphPad 3.00) was used. Differences at $p < 0.05$ were considered statistically significant, while differences at $p < 0.01$ were considered statistically highly significant.

3. Results

3.1. Imiquimod and Gardiquimod™ dose-dependently induce IFN-γ and IL-6 in thymocytes of naïve wild-type C57.BL/6 mice, but not IL-17

Thymocytes from female C57.BL/6 mice were stimulated with different concentrations of Imiquimod and Gardiquimod™ and cytokine production was assessed with ELISPOT analysis. As depicted in Figs. 1A and 2A, Imiquimod triggers IFN-γ production statistically highly significant at the concentration 0.5 μg/ml (mean: 58.8 SD: 52.3) and IL-6 production at the concentrations 5 μg/ml (mean: 92.9 SD: 31.8) and 0.5 μg/ml (mean: 91.7 SD: 49.2). Figs. 1B and 2B show that Gardiquimod™ has a stronger effect on IFN-γ and IL-6 production than Imiquimod. At a range of 5 μg/ml (mean: 96.1 SD: 47.4) to 2.5 μg/ml (mean: 123.9 SD: 55.6) Gardiquimod™ induces statistically highly significant frequencies of IFN-γ. For IL-6 statistically highly significant frequencies could be measured from a concentration range of 5 μg/ml (mean: 97.5 SD: 29.1) to 0.25 μg/ml (mean: 93.9 SD: 32.7) with the highest response at 2.5 μg/ml (mean: 141.9 SD: 43.6) Gardiquimod™. As indicated in Fig. 3A and B, no IL-17 production was induced either by Imiquimod or Gardiquimod™ alone.

3.2. Imiquimod and Gardiquimod™ potently co-stimulate cytokine production upon α-CD3 activation

To study the influence of TLR7 stimulation on the α-CD3-induced cytokine response, thymocytes from female C57.BL/6 mice were stimulated with 1 μg/ml α-CD3 and additionally with different concentrations of Imiquimod and Gardiquimod™. Overall, α-CD3-induced cytokine production was strongly enhanced, an effect which was statistically highly significant for all cytokines investigated. As seen in Fig. 1A, an increase of the frequencies of IFN-γ producing cells was detectable at a concentration of 0.5 μg/ml (mean: 338.1 SD: 137.2) Imiquimod. 5 μg/ml (mean: 53.7 SD: 65.7) and 0.05 μg/ml (mean: 67.0 SD: 55.9) Imiquimod showed only (statistically not significant) lower responses for IFN-γ production. As indicated in Fig. 2A, a statistically highly significant effect on the frequencies of IL-6 producing cells could be detected at 5 μg/ml (mean: 92.9 SD: 31.8) and 0.5 μg/ml (mean: 91.7 SD: 49.2) Imiquimod. As depicted in Fig. 3A, the IL-17 response was strongly enhanced at a broad concentration range: at the concentration of 5 μg/ml (mean: 42.3 SD: 20.2) Imiquimod, we could measure a statistically significant increase of the frequencies of IL-17 producing cells, concentrations of 0.5 μg/ml (mean: 61.5 SD: 23.8) and 0.05 μg/ml (mean: 49.0 SD: 21.2) Imiquimod even lead to a statistically highly significant increase compared to single stimulation with α-CD3 (mean: 16.2 SD: 8.6).

A similar pattern of co-stimulation could be detected for Gardiquimod™. As seen in Fig. 3B, a strong positive effect on the frequencies of α-CD3-activated IFN-γ-producing cells could be detected for 5 μg/ml (mean: 449.8 SD: 116.4), 2.5 μg/ml (mean: 507.1 SD: 69.1) and 0.25 μg/ml (mean: 369.4 SD: 102.4) Gardiquimod™. In regard to IL-6, concentrations of 0.25 μg/ml (mean: 333.1 SD: 91.6), 2.5 μg/ml (mean: 387.0 SD: 85.5) and 5 μg/ml (mean: 331.7 SD: 76.2) Gardiquimod™ induced a statistically highly significant increase.

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