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Role of TLR ligands in epicutaneously induced contrasuppression

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Abstract:
Our previous work showed that epicutaneous (EC) immunization in mice with protein antigen (Ag) induced an Ag-independent unresponsiveness mediated by suppressor $CD4^+8^+$ T cells (Ts), which inhibited contact hypersensitivity (CS). Simultaneous EC immunization with Ag and various Toll-like receptor (TLR) ligands reversed skin-induced suppression. Our present study shows that this process activates Ag-specific T contrasuppressor (Tcs) cells and leads to the protection of CS effector T cells from suppression. Epicutaneous immunization with Ag and the TLR4 ligand lipopolysaccharide (LPS) led to a significant increase in IFN-γ production by lymph node and spleen cells. Ag and TLR ligands, like LPS, CpG or lipoteichoic acid did not need to be applied concomitantly to the skin. An identical contrasuppressive effect was observed when the Ag and TLR ligands were deposited on distant skin areas, suggesting that both the generation of Ts and Tcs are independent. To corroborate this finding, we used a model system that uses macrophages (Mf) as Ag-presenting cells. Mf labeled *in vitro* with Ag (Mf-Ag) induced, upon intravenous (*iv*) administration, an unresponsiveness reaction that was mediated by Ts cells. When treated simultaneously with LPS-treated Mf (Mf-Ag-LPS), a TLR-ligand could induce CS. Both the Ag and the LPS signal could be uncoupled i.e., Mf-Ag and Mf-LPS given at separate time points (with an 1 h interval between injections) induced immunity. We also found that LPS-treated Mf also produced significant amounts of IL-12, a cytokine that has well-known anti-tolerogenic properties. Our experiments suggest that reversal of EC-induced suppression by TLR-ligands may be a potential tool to increase the immunogenicity of weakly immunogenic antigens.

Key words:
epicutaneous immunization, macrophages, Toll like receptor, reversal of suppression, contrasuppression

Abbreviations: Ag – antigen; APC – antigen presenting cells, CIA – collagen induced arthritis, CpG – oligodeoxynucleotide, CS – contact sensitivity, EAE – experimental autoimmune encephalomyelitis, EC – epicutaneous, FCS – fetal calf serum, IFN – interferon, Ig – immunoglobulins; IL – interleukin; *iv* – intravenous; LC – Langerhans cells; LNC – lymph nodes cells, LPS – lipopolysaccharide, LTA – lipoteichoic acid, MBP – myelin basic protein, Mf – macrophages, OVA – ovalbumin, PAMP – pathogen associated molecular pattern, PBS – phosphate buffered saline, Spl – spleen, Tc1 – T cytotoxic cell type 1, Tcs – T contrasuppressor cell, Th1 – T helper cell type 1, TLR – Toll-like receptor, TNP – 2,4,6-trinitrophenyl, TNP-Cl – 2,4,6-trinitrophenyl chloride, Ts – T suppressor cells

Introduction

Both the skin and mucosa are constantly exposed to many antigens and play a crucial role in protecting the body from a variety of distinct pathogens. The development of an immune responses to innocuous antigens is, at best, not helpful and often leads to a harmful allergic reaction. It is well known that immunization with an antigen (Ag) *via* the digestive tract or nasal mucosa leads to both a local immune response and to a state of profound immunosuppression at the periphery [27]. This suppression seems to play an important role in the attenuation of immune responses towards non-pathogenic Ags.

While the skin is considered an organ where immune responses are easily induced [2], little attention has been given to skin-induced tolerance [19]. Because the skin and the mucosa have a similar function in our bodies (i.e., as a barrier to external pathogens), it is possible that epicutaneous (EC) application of Ag, in addition to inducing a strong immune response, may also induce peripheral tolerance.

Indeed, our previous work showed that similar to the mucosa, EC immunization of mice with different protein Ags applied to the skin (in the form of a patch or cream emulsion) induced a state of subsequent Ag-independent suppression, a process mediated by suppressor T cells (Ts) that inhibit contact hypersensitivity [18]. This suppression was transferable *in vivo* by $\alpha\beta$-TCR $CD4^+$ $CD8^+$ double positive lymphocytes harvested from lymphoid organs of skin patched animals and was mediated *via* TGF-β [22].

We found similar results in an animal model of multiple sclerosis (EAE) and collagen-induced arthritis (CIA) where EC immunization with myelin basic protein (MBP) or collagen reduced disease severity and disease incidence respectively [12, 23–25]. Furthermore, our work employing allogeneic skin grafts showed that EC immunization with a protein antigen delayed graft rejection [11].

Recently, we showed that EC immunization with protein antigen TNP-Ig and Toll-like receptor (TLR) ligands reversed skin-induced suppression [16]. This finding suggests that EC immunization with an antigen in combination with TLR ligand administration might play an important role in immunopotentiation. Such a maneuver may potentially be effective for new vaccines and anti-cancer therapy.

The aim of current work was to precisely determine the mechanism of EC induced reversal of skin-induced suppression (immunopotentiation, contrasuppression).

Materials and Methods

Mice

Male CBA/J mice 6–8 week-old were obtained from the breeding unit of the Department of Human Developmental Biology, Jagiellonian University, College of Medicine. Mice were fed autoclaved food and water. All experiments were conducted according to guidelines of the Jagiellonian University College of Medicine.

Reagents

2,4,6-Trinitrophenyl chloride (TNP-Cl) was purchased from Chemica Alta (Edmonton, Canada). Ovalbumin (OVA, Grade V) and LPS (from *Escherichia coli* 026:B6*)* were obtained from Sigma Chemical Co. (St. Louis, MO). The CpG oligodeoxynucleotide TCCATGACGTTCCTGACGTT was prepared by the HHMI/Keck Oligonucleotide Synthesis Facility at Yale University. Lipoteichoic acid (LTA) from *Staphylococcus pulvereri* was extracted as described [6, 9], then fractionated on a gel filtration column (Bio-Gel P100) with phosphate buffered saline (PBS) as the eluant. LTA was donated by Dr. A. Gamian from the Institute of Immunology and Experimental Therapy, Polish Academy of Science, Poland.

Mouse immunoglobulins (Ig) were prepared from CBA/J mouse sera and conjugated with TNP hapten [8, 13]. A single preparation of conjugate with a ratio of 40 TNP molecules per Ig molecule (TNP_{40}-Ig) was used throughout the study. To measure the levels of IL-12p40 and IFN-γ in culture supernatants, mouse IL-12p40 and mouse IFN-γ ELISA kits were used (BD Pharmingen, San Diego, CA).

Additionally, horseradish peroxidase conjugated to streptavidin (Vector Laboratories, Burlingame, CA), and *o*-phenylenediamine, hydrogen peroxide (Sigma, St. Louis, MO) were used.

Epicutaneous immunization with TNP-Ig and TLR ligands

EC immunizations were performed by applying to the shaved skin of the mouse dorsum a 1-cm^2 gauze patch