Vaccine-induced Cytokine Production Detected by Luminex Multiplex Analysis

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[Abstract] Different vaccine and adjuvant combinations are known to rapidly induce antigen presenting cell (APC) maturation and pro-inflammatory cytokine and production, which in turn play an important role in the priming of antigen-specific T cells. Measuring cytokine production systemically in the serum fails to detect localized responses in the lymph nodes draining a subcutaneous immunization site. On the other hand, stimulating APC with vaccine formulations *in vitro* lacks the complexity of the lymph node microenvironment and the presence of other *in vivo* factors. Here we analyse cytokine production directly in vaccine draining lymph nodes (dLN) extracted early after *in vivo* vaccination. To do this we perform cytokine multiplex analysis of supernatants from whole dLN cell suspensions following a brief *ex vivo* incubation.

Materials and Reagents

1. C57BL/6 mice from Harlan Laboratories (the Netherlands)
   Note: All mice used in this protocol were between 6 and 12 weeks of age and were sex and age matched for each individual experiment. Three mice were used per group in each experiment.
2. OVA257-264 and OVA323-339 peptides
   Note: Peptides were manufactured by the Protein and Peptide Chemistry Facility (PPCF) of the University of Lausanne.
3. Iscove's modified Dulbecco's medium (IMDM), GlutaMAX™ supplement (Life Technologies, Gibco®, catalog number: 31980-030)
4. Penicillin-Streptomycin (5,000 U/ml) (Life Technologies, Gibco®, catalog number: 15070063)
5. 2-Mercaptoethanol (2-ME, 55 mM) (Life Technologies, Gibco®, catalog number: 21985-023)
6. Fœtal bovine serum (FBS) (performance sera with low endotoxin: qualified, US origin) (Life Technologies, catalog number: 26140 or similar)
7. Phosphate Buffered Saline (Laboratorium Dr. Bichsel AG)
8. Poly (I: C) HMW (tlrl-pic) and Imiquimod R837 (tlrl-imq, InvivoGen)

9. CpG-ODN 1826 (Coley Pharmaceuticals. No longer available. CpG-ODN 1826, tlrl-1826 from InvivoGen can be substitued)
10. Quil A saponin mix from Quillaja saponaria (Brenntag Nordic A/S)
11. Cytokine Mouse 10-Plex Panel for Luminex platform (Life Technologies, Novex®, catalog number: LMC0001) for use with the Luminex® 100™/200™ and FLEXMAP 3D® systems User manual available online: http://tools.lifetechnologies.com/content/sfs/manuals/LMC0001_Protocol_Rev1.pdf
12. Complete iscove's modified Dulbecco's medium (cIMDM) (see Recipes)

Equipment

1. 1 ml BD Tuberculin Syringe & 26 g or 27 g x 0.5" BD™ PrecisionGlide needle (Beckton Dickinson, catalog numbers: 3052111 or 305109)
2. Falcon™ 24 well, non-treated, flat-bottom tissue culture plates (Corning, catalog number: 351147)
3. ~40 µm nylon gauze sheets cut into small squares, *e.g.* Nylon Mesh Lab Pak, 41 Microns Square Opening (Nitex, catalog number: 7050-1220-000-14) or Nylon 6 Mesh Sheet, 48 microns Mesh Size (Small Parts, catalog number: B0043D1SCE)
4. Plunger from 1ml BD™ Tuberculin Syringe (BD, catalog number: 309602)
5. Dissection scissors and fine-nosed forceps or tweezers
6. Falcon™ 96 well, non-treated, U-bottom tissue culture plates (Corning, catalog number: 351177)
7. Luminex® 200™ System with xPONENT® Software (Life Technologies, Novex®)

Software

1. xPONENT® software (Life Technologies, Novex®)
2. Statistical tests were performed using GraphPad Prism software
   Note: The different groups were compared using One-way ANOVA with the Dunnett multiple comparison test, comparing all groups to the peptide alone group.

Procedure

A. Experimental protocol

1. C57BL/6 mice were immunised with 10 µg $OVA_{257-264}$ and 10 µg $OVA_{323-339}$ peptides in 100 µl PBS subcutaneously at the base of the tail: Briefly, mice were restrained in a holding tunnel with an opening at the end to access the base of the tail. About half the

length of the needle of the tuberculin syringe was inserted into the subcutaneous layer of skin just above and to the side of the tail. The correct depth can be confirmed by visualizing the bevel just below the skin. The vaccine is slowly injected while keeping pressure at the needle entry point. A bubble of liquid should become visible under the skin. The needle should be withdrawn carefully and the exit point massaged slightly with the fingertip to prevent escape of the vaccine.

2. Peptides were injected alone or in combination with 50 µg of one of the following adjuvants: CpG-ODN (CpG), HMW Poly (I: C), imiquimod, or Quil A.
3. Either 12 or 24 h later, mice were euthanised by CO_2 asphyxiation followed by cervical dislocation, and dLN (both inguinal LN) were excised.
4. LNs were rapidly transferred to a 24 well plate containing 250 µl of pre-warmed cIMDM + 5% FBS over a small square of 45 µm nylon gauze. A second piece of gauze was placed on top of the LN tissue, which was then dissociated by gently crushing with the rubber end of a 1 ml syringe plunger, while holding the mesh squares in place with forceps.
5. The cell suspension was then immediately pipetted into a 96 well u-bottom plate for incubation at 37 degrees for 1, 6 or 12 h.
6. The plate was centrifuged at 300 *x g* for 1 min before removing 65 µl of supernatant and freezing it at -20 degrees for later analysis.

   Note: Samples collected from multiple time-points for combined analysis should always be frozen rapidly to preserve the cytokines. However, analysis can be performed immediately if only a single time-point is to be analysed.

B. Luminex analysis

1. Cytokine production was measured with the mouse 10-plex Luminex panel [granulocyte macrophage colony–stimulating factor (GM-CSF), IFN-γ, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70 and TNF-α], and analysed using the Luminex 200 system with xPONENT software.
2. On the day of analysis, samples were thawed at room temperature.
3. The protocol outlined in the Luminex manual was followed exactly.
4. Standards were reconstituted with a mixture composed of 50% assay diluent and 50% of cIMDM.
5. 50 µl of thawed sample was added to the luminex plate along with 50 µl of assay diluent during the analyte capture phase.
6. The samples were run on the Luminex® 200™ system with xPONENT® software according to the manufacturer's instructions.

Recipes

1. Complete medium (cIMDM)
   IMDM
   100 U/ml Penicillin & 100 µg/ml Streptomycin
   55 µM 2-ME
   5% FBS

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References

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