MIIM30015 – Techniques in Immunology
Full Lecture and Practical Notes

Contents

Unit 2 Introduction: ELISA

PRRs

The innate immune system detects **PAMPs or DAMPs** that are foreign, through PRRs on innate immune cells

- These PRRs are **compartmentalised**
    - Cell surface
        - TLR
    - Endosomal
        - TLR
    - Cytosolic
        - NLR
        - RLR
- Compartmentalization is important as pathogens can invade cells at various locations. The PRRs help to **inform about the location of infection,** to **induce an appropriate response.** Urgency of the response is dependent on which compartment the pathogen is in.

Signalling functions

Depending on which TLR senses the PAMPs, there will be **various activation pathways** for the right response to be produced.

- TLRs 1,2,5,6
    - Use adaptor protein MyD88
    - Activate NFkB and AP-1 transcription factors
        - Eg. LPS and PGN PAMPs
- TLR3
    - Use adaptor protein TRIF
    - Activates IRF3 and IRF7 transcription factors
- TLR4
    - Can activate both pathways
- TLR7,9 in endosome
    - Use MyD88 adaptor
    - Can activate both NFkB and IRF7

The two outcomes are acute inflammation and stimulation of the **adaptive immunity (1)** and **antiviral state (2).**

- NFKB pathway
    - Expression of **inflammatory genes**
        - Cytokines (TNF, IL-1, IL-6)
        - Chemokines – CCL2, CXCL8 (mobilises cells like neutrophils)
        - Endothelial adhesion molecules – E-selectin
        - Costimulatory molecules – CD80/CD86
- IRF pathway
    - Expression of **Type I IFN (IFNaB) genes**
    - Secretion of Type I IFNs

Inflammasomes

Signalling via various PAMPs and DAMPs bound to PRRs will stimulate the expression of Pro-IL-1B. This then needs to be made active through the **formation of an inflammasome.**

- IL-1B is highly inflammatory so there are **secondary signals** to activate it
    - Mechanism of **better control**
- **Danger signal** received as well as **PAMPs** binding to the PRR
    - Eg. pathogenic bacteria, toxins, crystal formation, mitochondria dysfunction, K+ efflux
    - Extracellular ATP is a big danger signal as it is not normally there
        - **Leads to the formation of inflammasome complexes**
- Activated inflammasome process pro-IL-1B to active IL-1B
    - Inducing acute inflammation
    - Eg. NLRP3 inflammasome
        - Activates caspases which cleave the inactive molecules
- **Cell death occurs**
    - Likely **pyroptosis** as this is inflammatory
        - Alerts the other cells
    - Apoptosis is non-inflammatory
- Caspase function:
    - Activate pro-IL-1B
        - Then released as an inflammatory cytokine – IL-1B and IL-18
    - Produce cytotoxic pore forming proteins
        - Cause membrane rupture, cell swelling
    - DNA fragmentation

Dendritic Cells

Once you stimulate a D cell, it will then **present antigen to T cells** and activate the adaptive response. PRRs on the D cell recognise PAMPs and work to **produce cytokines** that act as the third signal for activation of the T cell, polarising the T cell response.

D cells also **upregulate certain inflammatory genes**/markers on the surface of the MHC – CD80/CD86 and CD40. These bind to the T cell and work to activate it or polarise it to help the T cell exert the right effects.

- D cells are **hard to isolate and purify** in animal models
    - We **don't get them in high numbers** to use in experiments
- In the lab we use **MutuDC cells**
    - Originate from the **spleen**
        - Originate from **conventional CD8a D cell subsets**
    - **Mouse** tumour derived cell line
    - Can undergo cross presentation
- MutuDCs are used in models to study cDC (CD8a) responses to activation with PRR agonists

Experimental setup and techniques

Aim: To investigate the effect of PRR mediated stimulation of activation of D cells

Method:

- Dendritic cell line **cultured in vitro**
- **Stimulate** with PRR agonists
- Read out activation of D cells by:
    - **Measuring cytokine production using ELISA (2A)**
    - **Measuring gene transcription using quantitative PCR (2B)**
    - **Measuring upregulated expression of stimulatory molecules using flow cytometry (2C)**
- We will stimulate mutuDCs for 4 or 18 hours with:
    - **LPS** – binds to TLR4
    - **CpG DNA** – TLR9 in endosome
    - **LPS + EC ATP** – inflammasome
    - **ATP** – as a control
    - **Unstimulated cells** – to compare our output to non-manipulated levels
- Measure:
    - **Cytokines by ELISA** – IL-1B, IL-6, IL-12, TNF
    - **mRNA expression** – NLRP3, IL-6, pro-IL-1B, B actin
    - **Costimulatory molecule expression** – CD86, CD40, MHC II

ELISA

- **ELISA can be used to measure cytokine secretion**
    - Checking for IL-1B, IL-6, IL-12 and TNF
- Sandwich ELISA method (Enzyme-linked immunosorbent assay)
    - Ab linked to an enzyme to give an output in an assay

- Process:
    - Set up a plate
    - In each well we have an Ab specific for the cytokine we want to assess **(capture Ab)**
    - Then we wash to get rid of unbound Abs
    - Then use **blocker** to fill up spaces on the bottom of the well to prevent non-specific binding
    - Then add the sample
        - The **sample is the SNF** from the cells we stimulated
    - Then do a wash to **remove things that are not of interest**
        - Only the cytokine binding to the Ab will remain
    - Then add another **Ab with biotin** on it
        - This will bind to the cytokine – a different epitope
        - Biotin is good at being detected by streptavidin
            - Streptavidin will detect this biotin
    - **HRP is on the streptavidin**
        - This is the enzyme which will produce the colour change
    - Then **add the substrate** (TMB) which will turn the well a different colour

Note: adding multiple layers of the antibodies increases the sensitivity because there are more things binding, so there is more signal amplified.

mRNA quantification using rt RT-PCR

We can use rt RT-PCR to **quantify the gene expression** of mRNA

- Abbreviations:
    - PCR = polymerase chain reaction
    - Q-PCR = quantitative PCR
    - (q)rt-PCR = quantitative real time PCR
    - (q-rt-)RT-PCR = quantitative real time Reverse Transcriptase PCR

When the D cell is stimulated, it will upregulate transcription and production of mRNA. Then we use this as starting material to produce cDNA, then this can be amplified in PCR. **Depending on how much mRNA there is = level of fluorescence when you amplify.** Therefore, more fluorescence = more transcription of a certain gene.

Process:

- Dendritic cells (unstimulated, +LPS, +CpG, +LPS and ATP, +ATP)
- **Extract their mRNA** (all of it)
- **Prepare cDNA** (using reverse transcriptase)
- Use the **cDNA as a template** in real time PCR amplification
- Examine levels of NLRP3, IL-6, pro-IL-1B and B-actin
    - **B-actin is a housekeeping gene whose levels should remain the same**
        - So this helps us to ensure our results are reliable

SYBR green qPCR method

- Have a **primer** that can detect your template
    - It will anneal based on the sequence being matched

- Then **polymerase** will extend the strand
- Then **intercalating dye** works to get in between dsDNA
    - When it does this, it **fluoresces**
        - As you create the products (amplicons) you get **more dye intercalated** so you can measure fluorescence

Results:

- Get result curves produced
- Cycles = how many times we have amplified from original template + fluorescence on y axis
- We see that the DNA will be copied through the PCR and you get more of it and then **more DNA = more fluorescence due to more dye getting in.**
    - Can measure in rt the amplification of dna into multiple copies as it is created

The housekeeping shouldn't change at all regardless of stimuli. **The target genes will amplify, and if there is a higher amount of starting material (mRNA), you get amplification earlier in the cycles, indicating that genes are upregulated more highly, producing more mRNA.** A lower starting material takes more cycles to amplify to detectable levels.

- **One curve should be seen** for the reference gene and target gene
- As you increase the temp, it will denature the DNA
    - The green will then disappear as it can't intercalate
    - So you lose fluorescence
        - Indicates you have one amplicon being made – one thing being amplified.

Flow cytometry analysis

We can use flow cytometry to do an analysis of surface molecule expression.

We will perform a surface stain with Abs against:

- CD11b – not upregulated. A marker on different WBCs
- CD11c – expressed on the D cell surface all the time – a marker for the whole population of cells
- MHC II
- CD40
- CD86

Practical 2A

Examining innate D cell stimulation by measuring cytokine production using ELISA

Introduction

- D cell stimulation of T cells requires 3 signals:
    - **TCR binding to MHC + peptide**
    - **Costimulation**
    - **Cytokines**
        - Cytokines released dependent on which PRRs are engaged on the DC
- TLRs are a subset of PRRs
    - Located on endosome and cell membrane
    - Example:
        - TLR4 recognises LPS
            - IC signalling for the expression of inflammatory genes occurs
            - Can also activate the antiviral state
        - TLR1,2,4,5 only activate the inflammatory genes
        - TLR3 will only activate the antiviral response
            - **Response is dependent on which TLR is engaged and which signal is received**
        - CpG DNA is recognised by TLR9 which is in the membrane of the endosome
            - TLR9 induces expression of inflammatory genes.
- NLRs are another subset and are in the cytosol eg. NLRP3
    - Requires recognition of an EC PAMP (such as LPS)
        - Engages TLR4
        - Induces the transcription of pro-IL-1B
    - Then PAMPs or DAMPs in the cytosol activate the NLRP3 inflammasome, including ATP
        - Then adaptor protein ASC recruits **Caspase1**
            - This cleaves pro-IL-1B into IL-1B
            - Other proteins form pores in the membrane causing pyroptosis
                - Inflammatory cell death

Pyroptosis

Pyroptosis helps to prevent replication of the pathogen and stops spread to other cells. The cell can eliminate IC replication of the pathogen and the cell eliminates the infection. The consequence is cell death and the release of inflammatory signals. The release of inflammatory cytokines recruits more immune cells and activates them to try and eliminate the IC pathogen that is expulsed during pyroptosis.

We do not see the production of IL-6, IL-12 and TNF in the presence of ATP – these cytokines are made during LPS alone but when we add ATP they are not produced.

Experimental Set Up

- Dendritic cells can be hard to study
    - Are present in **low frequencies** in vivo so are hard to isolate
    - **Fragile** and **sensitive** and have a **short life span**
- So we use a mouse tumour derived cell line – **MutuDC** used in this Prac
    - These cells are well characterised and **express GFP.**
    - They respond to TLR3 and TLR9 ligation but not TLR7
    - This cell line helps us study conventional D cells. They are + for CD8a
        - Originate from the spleen
        - Resident, non-migrating D cells that effectively cross-present exogenous cell-bound and soluble antigens
        - Upon activation, they produce IL-12 and stimulate inflammatory responses

Simulating conditions:

- Unstimulated
- LPS stimulated – 1ug/mL
- LPS+ATP - 1ug/mL LPS + 5mM ATP
- CpG alone – 1ug/mL
- ATP alone – 5mM

Culture for 4hrs or 18hrs and then collect the SNF and determine the concentration of cytokines by ELISA – IL-1B, IL-6, IL-12 and TNFa

Aim: To characterise if PRR stimulation led to cytokine production by D cells.

Method:

- Keep the cells inside a flask and keep the flask in an incubator with the proper conditions for cell growth
    - Each flask is assimilated with the 5 conditions for 4hrs or 18hrs
- Collect cell culture into tubes
- Then centrifuge to collect the cells at 1500rpm for 4 mins at 4C
- Then collect the SNF that is cell free
- Then quantify the concentration of the cytokines

In plate:

Materials: Antibodies, microtiter 96well plates, culture SNFs, standards (to determine the amount of cytokine and quantify it).

Abs and reagents being used:

- **Solid phase** – the plate which is **sticky plastic**
- **Capture Ab** – specific for the cytokine (eg. IL-6)
    - This binds to IL-6 at a specific epitope
- Then we **wash to get rid** non-specific binding.
- Add **blocking buffer**

- Then we add the SNF
    - In the SNF is the cytokine hopefully
        - There will be lots of cytokine and other proteins expressed by the cell
- Then we wash again
    - We are left with the specific cytokine only – IL-6
- Then we add a **secondary Ab** which is **conjugated to biotin** (biotinylated)
    - The secondary is targeted to diff epitopes on IL-6
        - The first epitope is bound to the primary epitope
        - This also **increases specificity of the assay**
- Then we do another wash
    - There is blocking agent in the wash
- Then we **add HRP** which is conjugated to streptavidin
    - There are multiple binding sites on streptavidin
        - Multiple HRPs are on it to multiply the signal
    - Now we have the HRP, this helps us to **visualise the reaction**
        - **The substrate we add is TMB** which will go from **clear to blue**
            - We keep it covered in the lab because it is also light activated
            - Will give high background reading if not in foil
- **Add TMB**
    - HRP will turn it to blue if there is IL-6 present.
    - Note: We do not wash after this step because otherwise we would wash away the substrate colour

We want to **stop the reaction** because HRP will keep performing the reaction until all the TMB has turned to blue. So we stop it happening by adding an **acid like HCl** to turn it yellow

- Then we can read it on a **plate reader**

Standards

<u>Determining the amount of cytokine in a sample</u>

- We will **need standards**
    - You measure the **colour density** by using the standards.
    - We refer to these to find the colour
- Reagents are **stored on ice** so they don't deteriorate
    - Streptavidin **HRP is light sensitive** so is in foil
- Standards are also stimulated for 4hrs and 18hrs
    - Standards are done twice so that we can check that everything is working as it should

<u>Other materials and equipment</u>

- **Diluent dilutes the substrates** and things we will add
- Pipette – multichannel (8 wells) and single pipette
    - Set them to diff volumes to use

Each cytokine standard has a diff starting dilution – we will do 2 fold serial dilutions of these. We run the standards in duplicate because if they are both the same we know they are correct.

We incubate for 60 minutes with the antibody to give it enough time to bind, but with a blocker to prevent non-specific binding. This is because the plate contains high binding plastic, so if it is exposed, proteins may bind non-specifically.

Lab live stream

- 4 diff ELISA plates – one for each cytokine (IL-6, IL-1B, TNFa, IL-12)
    - 96 wells in each plate
    - **The Cytokine Standard is a recombinant protein of the cytokine**
    - Everything secreted from the D cell (SNF) is collected and in the tubes
- The plates are already coated with **capture Abs**
- **Blocking solution** added
    - Contains a protein – so nothing else can bind to the sticky plastic
        - **BSA** is the protein
- We also do a **wash** to get rid of the unbound antibodies
    - We wash with PBST
        - Phosphate buffered saline with tween
            - A mild detergent
- Add 50uL **diluent** into the seven wells in column 1 and 2 of standards (not the top well)
    - Diluent is the same media that the cells have been growing in
- Add 100ul of the standard in the first two wells (without diluent)
- Then do 50uL **serial dilutions**
    - Take 50uL out of the first well and put it in the second well
        - The type of serial dilution is 2 fold.
    - If the first well is 2000pg/mL, the second well is 1000ug/mL
        - End up with 100uL in the last well
            - Now need to take out 50 and discard it
                - We need to do this to have equal vol in each well
        - There is 0 standard in the 0 wells
            - There will still be background control but no standard
                - In these wells there should be no colour change
                - No standard cytokines were added
                    - It helps us see for background optical density
- We then **add the samples** into their wells - 4hr and 18hr
    - Diff stimulation conditions
    - Do not scrape the bottom of the wells
- Then we need to **incubate** for 15mins
    - This gives time for the cytokines to bind to the Ab
        - Incubation time depends on the concentration of the reagents
            - If you incubate for a short amount of time, you wont get the binding occurring
            - If you incubate for too long it wont make a big diff at this stage
                - But if HRP is on for too long, it increases the background
- The antigen will be recognised by the Ab and capture it