Formative assessment

Course	Immunology
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Overall background

Aberrant T cell activation is a driver of pathology in various T cell-mediated autoimmune disease (e.g. Type I diabetes, multiple sclerosis, and rheumatoid arthritis).

Inflammatory cytokine production by myeloid cells is identified as the mediator of substantial pathology.

→ Trying to understand the mechanism cause the cytokine production

- Previous research targeting PRR (pathogen recognition receptors) in the disease demonstrate noninfectious nature of this inflammation

→ Other upstream pathway exists

Treg deletion ablation cause broad T cell activation as well as DC and macrophage activation

Cytokine storm of CAR T cell therapy has not been correlated to microbial recognition by PRR

- Transfer of activated antigen-specific T cells leads to systemic inflammation and elevated level of IL-6 in blood

    - IL-6 is myeloid origin

Tem were able to directly instruct de novo innate cytokine production by myeloid cels as part of a broad inflammatory program.

- Transcriptomic analysis revealed that this Tem-driven activation of myeloid cells overlaps substantially with PRR activation

→ Tem-derived cues essentially replace microbial ligands to drive innate immune activation

CD40 signalling is the primary trigger for PRR-independent myeloid cell activation by Tem with contribution from TNF receptor signalling

Blocking CD40 and TNFR pathways together completely abrogated the cytokine storm induced by activated Tem as well as autoimmune organ patogen associated with the presence of self-reactive T cells.

Findings

1. **Tem instruct myeloid cells to produce innate cytokines during their cognate interaction**

Cytokine productions was seen only when aCD3 is added (T cell signalling)

→Stimulation of T cells is required for the cytokine production

No difference when TLR genes are KO

→This production is independent of TLR (PAMPs)

When Il6 gene knocked out in BMDC, no cytokine production

CD11c+ and CD11b+ (BMDC) - cytokine production

CD90.2 (T cells) - no cytokine production even T cell signalling present (aCD3)

→ Source of cytokine is from BMDC

2. **Tem-instructed and PRR-mediated activation of myeloid cells share a substantial transcriptional profile**

In the presence of activated Th cells, there is increased expression of maturation markers such as CD86 and MHCII

→ DCs are activated by activated Th cells

Some of the genes that are upregulated or downregulated are the same in anti-CD3 stimulated T cell cocultures and LPS stimulated DC and some are different.

- LPS stimulation causes more up and down regulated pathways

→ Activation of DCs in the co-culture is similar but not identical to the innate activation induced by the classic TLR/ LPS stimulation

3. **CD40 and TNFR signaling pathways are critical for PRR-independent activation of DCs by Tem**

- Anti-TNFa: block TNFR signalling
- Anti-CD40L: block CD40 signalling

Both anti-TNF and anti-CD40L reduce IL-6 production, Anti-CD40L effect > Anti-TNF

There is an additive effect when two blockade combined

- This result is confirmed both in vitro and in vivo

→ Both TNF and CD40 signals are important to induce optimal activation of DC

Addition of antibodies against TNFa and CD40L improves survival / prevents death induced by administration of antiCD3 antibody in vivo / prevents cytokine storm

- Using animals which are unable to produce (KO) TNF/ CD40 has the same effect: no cytokine storm is induced/ animals survive

→ TNFa signalling and CD40-CD40L interaction is key for the production of IL-6 (and other inflammatory cytokines)

MOG 35-55 peptide from myelin oligodendrocyte glycoprotein recognised by CD4 T cells

- MOG-imm tCD4: Immunised with MOG
- To mimic self antigen/ autoimmune disease setting (MS)
- In vitro co-culture but antigen specific stimulation of T cells

Blocking TNF and CD40L pathway results in reduced cytokine production

4. **Autoreactive T cells drive inflammatory cytokine production by DCs and macrophages in vivo**

Treg ablation by DT causes neutrophils, inflammatory monocyte, macrophages (CD11b+, CD11c-), and cDc (CD11c+) infiltration to the spleen

In the absence of Tregs IL-6 and IL-12 are increased in the serum

→ Conclusion: Tregs control activation of T cells when removed, over-active (self-reactive) T cells lead to activation of innate immune cells.

Generation of iTregs in co-cultures suppresses IL-6 production

- tCD4: CD4+ cells from Foxp3 DTR mice- activated in vivo
- iTreg: induced Tregs
- nCD4: naïve CD4 (not activated)

→ This confirms Foxp3+Tregs suppress activated Teff cells which would stimulate cytokine production by myeloid cells via the TNFa/ CD40L pathways

Effector memory T cells mobilise a broad pro-inflammatory program in the innate immune system.

- Instead of PRR activation of DCs, DCs are activated by Tem

Anti-CD40L has a stronger effect (decrease in cell number of neutrophils, monocytes, macrphages) when used on its own compared to anti-TNF on its own

Anti-TNF added in addition to anti-CD40L does not show any additive effect

→ Signalling through CD40-CD40L by T cells plays a crucial role in controlling myeloid cell
numbers (induced by T cells activated in vivo, due to the removal of Tregs)

In the absence of Tregs, production of IL-6 and IL-12 present in the serum is dependent on TNF and CD40L signalling

- Activation of different splenic myeloid cells is dependent on TNF/ CD40 signalling (as measured by CD86 expression
- IL-6 and IL-12 are produced by different myeloid cells (in the absence of Tregs) and reduced by anti TNF/ CD40L antibodies

Dendritic cells

Classical or conventional dendritic cells (cDC)

- Developmentally and functionally similar population, present in peripheral tissues and secondary lymphoid organs (a small population will also be present in circulation)
- Express `CD11c` as a lineage marker
- Based on the expression of various molecules (e.g. CD11b, CD103, CD8alpha, CD4) DCs can be divided into further subsets with different functions

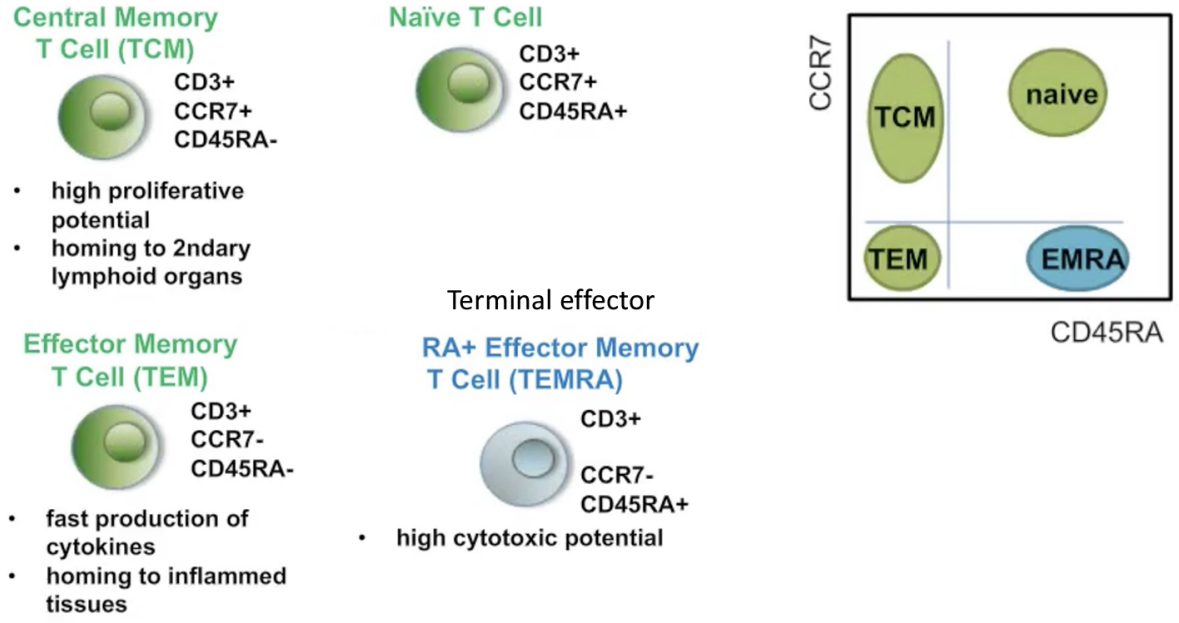
Activation of DC

- Immature DCs are not very good at antigen presentation and stimulation of T cells
- In response to PAMPS and inflammatory cytokines DCs mature and become excellent at activating naive T cells
    - Microbial products, inflammatory cytokines, damaged host cells (danger signals)
- Mature DCs express high levels of MHC Class II and co-stimulatory molecules such as CD80 and 86
    - They also express CCR7 which allows them to home to the draining LN
- Mature DCs migrate to LN via afferent lymphatics and present antigenic peptides to T cells (CCR7/CCL21)
    - Chemokines ensure them to the lymph nodes using the gradient of CCL21

T cell activation and memory

- In order for naive T cell to become activated they need to receive different types of signals from the APC
    - Signal 1 is the specific peptide, presented within the MHC molecule binding to the TCR
    - Signal 2 is co-stimulatory signal, the most important is provided via CD80/86 (on the APC) which interact with CD28 on the T cell
        - CD4 stabilise the interaction
        - DC provide signals for T cells BUT T cells also provide signals for DC
    - Signal 3 is cytokines secreted by APC (and other cells) can influence the development of functional subsets of T cells
        - May be IL-2 acting in an autocrine or paracrine function
- Once a naive T cell is activated it changes expression of certain surface molecules
    - Early changes: Increase expression of CD69 and CD25 (IL2R alpha)

- Naive T cells express CD45RA; activated T cels and memory T cells express CD45RO
- T cells change the expression of chemokine receptors and their migratory capacity
  - CCR7+ and CD62L+ T cells are able to migrate to the LN
  - Other chemokine receptors (CCR7-) allow migration to the tissues (e.g. CXCR3)
- Memory T cells are often subdivided into central memory and effector memory populations based on their expression of CCR7 and migration patterns



- CD3: T cell lineage marker
- CCR7: migration to the lymph node
- CD45RA
  - Usually upon activation, cells will not express regulating RA
  - TEMRA: highly differentiated, not much further proliferation, might accumulate with age or chronic infections
- Effector T cells and Effector memory T cells (TEM) are difficult to distinguish between

    - E.g. chicken pox infected 10 years ago or currently
    - Phenotypically identical, and become TEM once infection is cleared
- Effector CD4 T cells produce cytokines such as IL-4 (Th2), IFNgamma (Th1), IL-17 (Th17) and effector CD8 T cells express granzyme B or perforin and are termed CTLs (cytotoxic T lymphocytes)
    - During the stimulation of naive CD4 T cells, the presence of different soluble factors (cytokines) determines if the cells will be polarised into Th1, 2, or 17 effector cells.
    - The same process can be recapitulated in vitro by addition of relevant cytokine combinations
    - Th0 population is unpolarised, mostly produces IL-2, and can be considered an early effector population which may become more polarised over time

> 💡 Polarisation: the process immune cells adopt distinct programs and perform specialised functions in response to specific signals

- Treg: A specialised subset of CD4 T cells have regulatory (suppressive) function
    - Identified by expression of the transcription factor Foxp3, expression of CD25 and CTLA4 and secretion of inhibitory cytokines (TGFbeta and IL-10)
        - Separate lineage determined to be Treg when leaving the thymus, some can develop in the periphery by induction
        - Foxp3 drives Treg development, mutated Foxp3-lymph proliferative disease, can be lethal
        - Express CD25 (IL-2 receptor alpha), IL-2 is an important cytokine for T cell proliferation (survival)
        - CTLA-4 bind to CD80/86, prevent CD28 binding and T cell proliferation
            - Negative homologue of CD28
    - Control the immune response

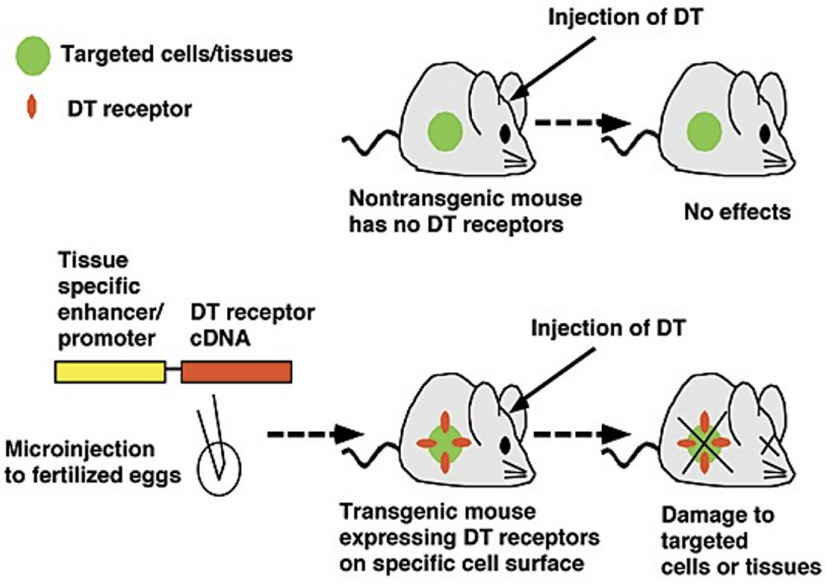
    - Depletion of these cells (by genetic mutation during development or experimentally) leads to inflammation and autoimmunity

Methodology

Congenic mice

- Congenic: Strains which differ genotypically only in one locus of particular genetic region
- CD45 and CD90 are common common antigens expressed in all leukocytes
    - Two different alleles, CD45.1 and CD45.2 (Ly5.1 and 5.2) and CD90.1 and CD90.2 (Thy1.1 and Thy1.2) are functionally identical and can be easily detected by flow cytometry using specific antibody
- In bone marrow transplantation experiments, congenic mice with CD45.1 versus CD45.2 alleles are often used
    - Allows adoptive transfer of cells (e.g. expressing CD45.1) into a host animal (CD45.2) and subsequent identification of donor cells based on the expression of this molecule (using flow cytometry)
    - Alternatively, cells with different congenic markers can be mixed in vitro and then their responses compared by “gating” on the congenic marker to identify them
- Congenic markers allow mixing of cells and tell them apart, especially in vivo
    - Transfer the two different cells to the same mouse to control condition

Diphtheria toxin receptor (DTR) technology



- Mouse cells don't usually express DTR unless they are engineered
- Allow specific cell ablation post-development at any chosen time (the cells that express DTR will be deleted in the presence of diphtheria toxin, DT)
- It is a useful method for analysing the in vivo function of specific cell populations
    - e.g. Foxp3DTR: cells expressing Foxp3 express DTR, thus will be deleted by DT.

Anti-CD3 antibody

- Antibody which binds to the CD3 co-receptor (part of the TCR complex) mimicking TCR stimulus in vitro

OT II transgenic mice / OT II cells

- Transgenic mice express the mouse alpha-chain and beta-chain T cell receptor (that pairs with the CD4 coreceptor) which is specific for chicken ovalbumin peptide 323-339; All CD4 T cells recognise this OVA peptide

MOG (myelin oligodendrocyte glycoprotein) 35-55 peptide

- Antigenic peptide that is used in induced experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis

TLR2/4/5xUnc93b1 (3d/3d)

- Lack TLR2, TLR4, TLR5, as well as the functional protein UNC93B1 responsible for TLR3, TLR7, TLR9, TLR11, and TLR13 trafficking so effectively lack ability to respond to any of the TLR signals

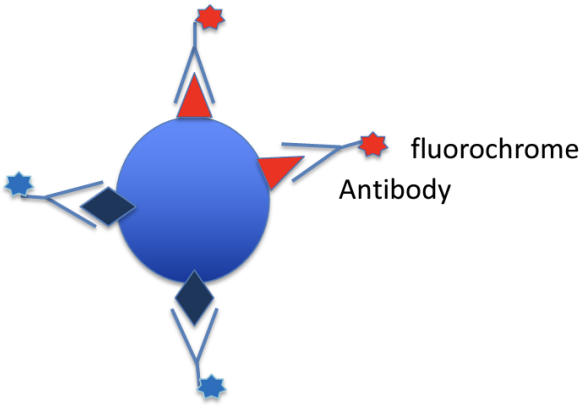
Immunofluorescence

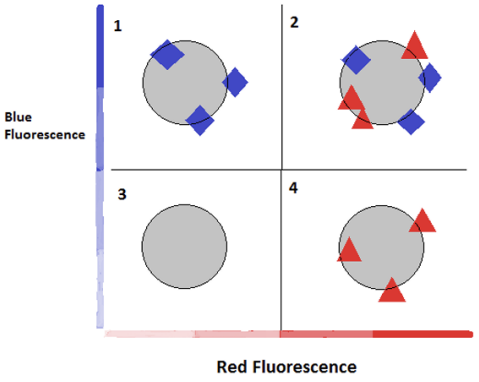
- Fluorescent molecules include: GFP (green fluorescent protein), RFP, phycoerythrin, tomato red

ELISA and serum ELISA

- A method for measuring the amount of protein in solution
- Can be used to measure cytokine secreted after stimulation
- Enzyme labelled antibody binds to protein of interest, substrate, colorimetric signal measured, plate reader
- Standard curve allow quantification

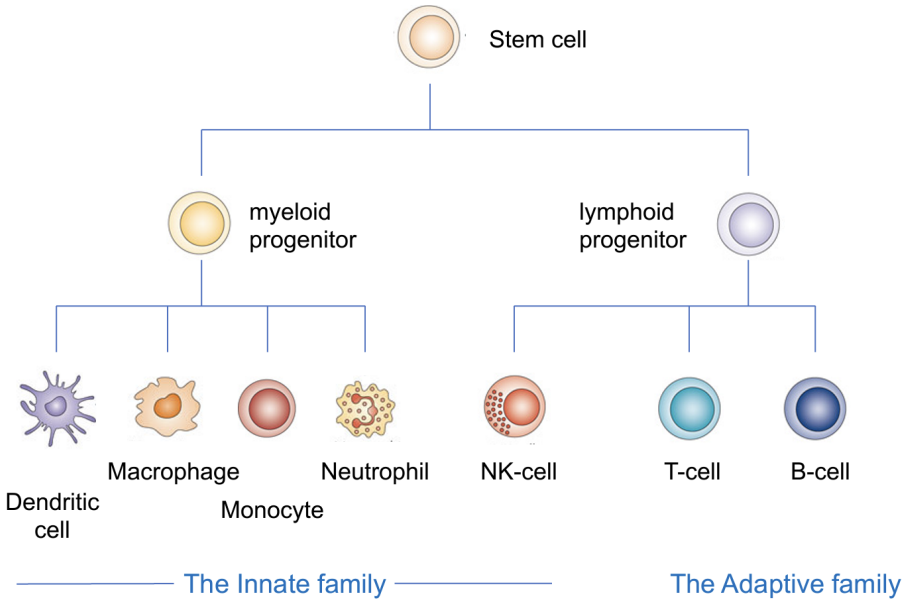
Flow cytometry and cell sorting





- Monoclonal antibodies against particular surface molecules
- Conjugated to fluorescent label/fluorochrome
- Flow cytometer - measures intensity of fluorescence of each cell
- Using controls to distinguish positive and negative populations
- 4 populations of cells can be determined by 2 antibodies

Percentage cytokine positive - flow cytometry; cytokine measured as concentration - ELISA



- Leukocytes: while blood cells (immune cells in blood)
- Lymphocytes: T cells and B cells and NK cells (innate)
- Granulocytes: Neutrophils, Eosinophils, Mast cells, Basophils