Section

1

General Pathology including Immunopathology

Cancer Invasion and Metastasis

1

Sakshi Sirswal

INTRODUCTION

- "Neoplasia" (*new growth* in Greek) is an abnormal proliferation of cells, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change.
- "Neoplasm"—an abnormal mass of tissue produced.
- Neoplasms can be benign, pre-malignant, or malignant (cancer).
- There are eight fundamental changes in cell physiology, which are considered the hallmarks of cancer:
  1. Self-sufficiency in growth signals
  2. Insensitivity to growth-inhibitory signals
  3. Altered cellular metabolism
  4. Evasion of apoptosis
  5. Limitless replicative potential (immortality)
  6. Sustained angiogenesis
  7. Ability to invade and metastasize
  8. Ability to evade the host immune response.

Overview of Carcinogenesis

- Carcinogenesis is a multistep process at both the phenotypic and the genetic levels, resulting from the accumulation of multiple mutations involving multiple genes.
- Phenotypically, malignancy is characterized by excessive growth, local invasiveness and ability to form distant metastases.
- The principal targets of genetic damage include:
  - Genes promoting cell growth (proto-oncogenes)
  - Genes inhibiting cell growth (tumor suppressor genes)
  - Genes regulating apoptosis
  - Genes regulating DNA repair.
- As the tumor ages, multiple mutations accumulate independently in different cells, generating subclones with varying abilities to grow, invade, metastasize, resist/respond to therapy.
- Most malignant tumors are monoclonal.

- However, by the time they become clinically evident their constituent cells are extremely heterogeneous.
- The ability of the tumor cells to metastasize is largely dependent on the ability of these cells to migrate.
- Hence an important step in carcinogenesis is epithelial-mesenchymal transformation that bestows the tumor cells with the abilities like invasion and metastasis.
- Fundamentally, the epithelial cells and mesenchymal cells have their own characteristics that allow them to behave differently.

Characteristics of Epithelial Cells

- **Epithelial cells** are cells that line the inner and outer surfaces of the body in continuous cell layers, as a single layer or multilayered structure.
- The epithelial cell layer is attached to the underlying connective tissue by a basement membrane.
- Epithelial cells are joined together by strong intercellular cell junctions that help in the maintenance of continuous cell layers.
- Epithelial cells are characterized by specialized membrane domains:
  - Basal domain
  - Lateral domain
  - Apical domain: Characteristics of this domain depend on the functional needs of the cell.
- **Lateral domain:** Keeps the epithelial layer impenetrable and consists of desmosomes, gap junctions, tight junctions and adherens junctions.
  - *Desmosomes:* Adhesion belts connected to intermediate filaments
  - *Gap junctions:* Intercellular channels 1.5–2 nm in size that permit free passage of ions and small molecules. Also, gap junctions permit changes in membrane potential.
  - *Tight junctions:* Bind cells with claudin, occludin proteins.
  - *Adherens junctions:* These are built from cadherins.
- **Basal domain:** Allows interaction between the basement membrane and the cell. The cells adhere to extracellular matrix (ECM) by:
  A. *Hemidesmosomes:* Bind the ECM to intermediate filaments via integrins.
  B. *Focal adhesions:* Bind the ECM to actin microfilaments via integrins.

Characteristics of Mesenchymal Cells

- **Mesenchymal cells** have the following characteristics:
  - They do not have cytoplasmic polarity.
  - They do not form a continuous sheet.
  - There are no specific membrane domains.
  - Intercellular adhesions are less strong which accounts for the migratory capacity of the mesenchymal cells.
  - Cells are mobile with a cytoskeleton comprised by vimentin.
  - The cells have filopodia as cytoplasmic extensions when mobile, which contain active cytoskeleton.
  - They express matrix adhesion proteins and matrix metalloproteinases (MMPs).
- Mesenchymal cells contribute to the ECM by synthesizing and organizing new components by remodeling the ECM through the production of matrix-degrading MMPs.

- Mesenchymal migration is mechanistically different from epithelial movement.
- Epithelial cells move as a sheet en block, whereas mesenchymal cells move individually.

INVASION AND METASTASIS

- Invasion and metastasis are hallmarks of malignant tumors.
- They are a major cause of cancer-related morbidity and mortality.
- The causes of metastasis can be explained using four models as follows:
  A. Metastasis is caused by rare variant clones that develop in the primary tumor.
  B. Metastasis is caused by the gene expression pattern of most cells of the primary tumor, referred to as a metastatic signature.
  C. Combination of A and B, in which metastatic variants appear in a tumor with a metastatic gene signature.
  D. Metastasis development is greatly influenced by the tumor stroma, which regulates angiogenesis, local invasiveness, and resistance to immune elimination, allowing cells of the primary tumor to become metastatic.

METASTATIC CASCADE

There are two phases of metastasis:
1. Invasion of the extracellular matrix (ECM).
2. Vascular dissemination, homing of tumor cells and colonization.

INVASION OF THE ECM

- Normally cells are glued to each other due to the presence of cell adhesion molecules, e.g. cadherins.
- Loss of E-cadherin is important in the development of almost all epithelial cancers
- The tumor cells and tumor stromal cells secrete proteolytic enzymes like—MMPs, cathepsins, urokinase and plasminogen activator.
- These proteases degrade basement membrane and interstitial connective tissue.
- Cleavage products of collagen and proteoglycans, thus formed, have chemotactic, angiogenic and growth-promoting effects.
- The loosened cancer cells bind to proteolytically generated binding sites, mainly laminin and fibronectin.
- The loss of integrins favors invasion.
- ***Locomotion*** is the final step of invasion.
- Cytokines released by tumor cells and cleavage products of matrix facilitate migration of tumor cells.

Vascular Dissemination, Homing of Tumor Cells and Colonization

- Once the cancer cells detach from neighboring cells, they migrate to surrounding tissue.
- The cancer cells then pass through blood vessels and enter the systemic circulation (intravasation).
- On reaching the metastatic site the tumor cells exit the bloodstream (extravasation).
- In this new site the tumor cells reconstitute the tissue environment which resembles that of a primary site and is favorable for the growth and survival of tumor.

- To develop migratory behavior, epithelial cancer cells acquire properties close to mesenchymal cells and this phenomenon is called epithelial-mesenchymal transition (EMT).
- EMT facilitates cell invasion and metastasis.
- However, these mesenchymal-like tumor cells gain migratory capacity at the expense of proliferative potential.
- To establish metastasis, the metastatic tumor cells undergo inverse process of mesenchymal-epithelial transition (MET).
- MET is required to regenerate a proliferative state and form macrometastases resembling the primary tumor at distant sites.

CONCEPT OF EMT

Historical Background

- Two main cell types—epithelial and mesenchymal were recognized in the late 19th century based on their shape and organization during embryonic development and interconversion between the two states was described by Frank Lillie in 1908.
- In the late 1960s, Elisabeth Hay provided a detailed description of the formation of chick primitive streak—a structure that requires the conversion of epithelial to mesenchymal cells.
- EMT was recognized as a distinct process in 1982 by the work of Garry Greenburg and Elisabeth Hay on the 3D culture of corneal epithelial cells in the laboratory.
- It took a long time for EMT to be recognized as a potential mechanism for carcinoma progression because of the following reasons:
  - EMT cannot be followed in time and space in human tumors.
  - Great diversity of cellular organization in human tumors.
  - Recognition of carcinomas (epithelial origin) and sarcomas (mesenchymal origin) as two separate entities, not thought to interconvert (except a rare tumor known as sarcomatoid carcinoma).
- The mechanisms that govern EMT are now being unraveled and many parallels are being found between EMT in embryonic development and EMT in tumor development.

Role of EMT

- Under physiological conditions, EMT plays an important role in embryonic development.
- EMT also plays a crucial role in pathological conditions like tissue reconstruction after injury (wound healing), chronic inflammation, tissue fibrosis in response to injury (lung, kidney, liver), carcinogenesis, tumor metastasis and invasion.
- However, the difference between normal development and pathological processes is at cellular and molecular level.
  - Events follow highly regulated spatial and temporal plans during physiological development.
  - During pathologic transformation, the order of events may be stochastic and time-independent or particular events may be bypassed.
- During tumorigenesis EMT may increase motility and invasiveness of cancer cells and malignant transformation may be associated with signaling pathways promoting EMT.

Epithelial Plasticity is Bidirectional

- The reverse phenomenon, back to epithelial phenotype is called, mesenchymal-epithelial transition (MET).
- MET occurs at various stages of morphogenesis, alternating with EMT during the formation of heart, somites, and kidney, and in the formation of coelomic cavities.
- ***Metastable phenotype*** refers to the ability of cells to express attributes of both epithelial and mesenchymal phenotypes.

E-CADHERIN AND EMT

- E-cadherin is a calcium-dependent transmembrane glycoprotein which functions as an adhesion molecule. It is present in most epithelial cells in embryonic and adult tissues.
- Both EMT and MET are dependent on E-cadherin.
- The cells undergoing EMT downregulate E-cadherin.
- In various human carcinomas, functional loss of E-cadherin may result from
  - Production of a defective protein
  - Promoter hypermethylation
  - Gene mutation
  - Transcriptional repression may result from the activation of repressors, such as Snail, Slug, Sip1, Ets, Twist.
- **Under physiologic conditions,** the signaling pathways that lead to cell proliferation, differentiation and migration begin with growth factor binding to a specific receptor.
- This is followed by activation of signal-transducing proteins which transmit the signal across cytosol to nucleus.
- This leads to induction and activation of nuclear regulatory factors which initiate DNA transcription.

Signaling Pathways in EMT

- Various classes of molecules that change in expression, distribution, and/or function during the EMT, and that are involved, include (Table 1.1):
  - Growth factors [e.g. transforming growth factor (TGF)-β, Wnt]
  - Transcription factors (Snails, SMAD, LEF, and nuclear β-catenin)

Table 1.1: Pathways involved in the regulation of EMT

Receptor	*Ligand*	*Signaling molecule*	*Intermediate signaling endpoint*	*Effect*
TGF-β receptor	TGF-β	RhoA	MRTF-A	Stress fibers migration
FGF receptor	FGF	Smad	SNAIL2	Cytoskeleton activation migration, focal adhesion, rearrangement
Tyrosine kinase	HGF	Sarc	SNAIL2	Increased migration
Integrins	EGF	Ras, MAPK	SNAIL1	E-cadherin downregulated, reduced cell adhesion
Frizzled	Collagen, fibronectin	Paxillin, Rac	GIT1	Stress fibers migration

- Molecules of the cell-to-cell adhesion axis (cadherins, catenins)
- Cell-to-ECM adhesion axis (integrins, focal contact proteins, ECM proteins)
- Cytoskeletal modulators (Rho family)
- Extracellular proteases (matrix metalloproteinases, plasminogen activators).
- Other molecular changes seem to occur after the initial behavioral change; for example, there is often a trend to replace cytokeratin intermediate filaments with other types, typically vimentin.

INDUCTION OF EMT

- The first event in EMT is the proteolytic digestion of the basement membrane by metalloproteinases.
- Local expression of TGF-β, EGF, IGF-II and FGF-2 facilitates EMT by binding to receptor with ligand-inducible intrinsic kinase activity.
- Overexpression of master regulators of EMT, such as the transcription factors like Twist, Snail, and SIP1 repress the expression of E-cadherin.

TGF-β Pathway or Smad Signaling Pathway

- TGF-β interacts sequentially with two membrane receptors.
- TGF binds first to the type II receptor and then the ligand-receptor complex associates with type I TGF receptor.
- TβIIR phosphorylates TβIR.
- Activated TGF-RI propagates the signal downstream by phosphorylating Smad2 and Smad3 which form complexes with Smad4 and translocate into the nucleus.
- In combination with T cell factor (TCF) family transcription factors, they down-regulate E-cadherin genes and initiate EMT.

Wnt/β-catenin Signaling Pathway

- Wnt signals through a family of cell surface receptors called frizzled (Frz).
- It stimulates several pathways, the central one involves **β-catenin** and APC.
- It regulates the amounts of β-catenin protein available within the cell for binding cadherins.
- It mediates
  - cell-cell adhesion
  - adhesion to cytoskeletal (F actin) elements
- In resting cells, β catenin forms complex containing glycogen synthase kinase3 (GSK3β), axin and APC protein.
- Phosphorylated β-catenin is degraded by ubiquitination.
- Hence, intracellular level of β-catenin is kept low.
- Wnt-Frz leads to dissociation and inactivation of GSK3β, which can no longer phosphorylate β-catenin.
- Free β-catenin translocates to the nucleus, thereby inducing gene expression in a complex with T cell factor (TCF) down-regulating E-cadherin and initiating EMT.
- For example, in APC-mutated in colon cancer, cells behave as if they are under constant stimulation by Wnt pathway.
- This leads to EMT induction as β-catenin translocates to nucleus.

Tyrosine Kinases Pathway

- Several growth factors can induce EMT by binding to receptor tyrosine kinases.
- FGF, EGF, TGF-α and IGF-2 can induce EMT.

TRANSCRIPTION FACTORS ASSOCIATED WITH EMT

Twist

- Twist transcription factors cooperate with mitogenic oncoproteins in cancer cells.
- Twist contributes to the malignant transformation via the following:
  - Overrides premature senescence (inhibits apoptosis).
  - Downregulates E-cadherin.
  - Upregulates mesenchymal markers like Vimentin, SMA.
  - Induces EMT allowing tumor progression and dissemination.

Snail

- Snail is a strong direct repressor of E-cadherin.
- It confers tumorigenic, invasive and migratory properties.
- It inhibits apoptosis.
- It inversely correlates with the degree of differentiation and is associated with lymph node metastasis.

Steroid Receptor Coactivators Interacting Protein (SIP)

- SIP, a novel ankyrin repeat containing protein, sequesters steroid receptor coactivators in the cytoplasm.
- It contains a Smad-binding domain and may therefore modulate TGF signaling pathway, which is known to induce EMT.

EMT MARKERS

- Table 1.2 gives a list of markers that can be used for diagnosing EMT.

Table 1.2: Markers for EMT

Proteins that increase in abundance	
N-cadherin	MMP-2
Vimentin	MMP-3
Fibronectin	MMP-9
Snail1 (Snail)	Integrin αvβ6
Snail2 (Slug)	N-cadherin
Twist	Vimentin
FOXC2	Fibronectin
Sox10	Snail1 (Snail)
Proteins that decrease in abundance	
E-cadherin	Cytokeratin
Desmoplakin	Occludin

Contd.

Table 1.2: Markers for EMT	
Proteins that decrease in abundance	
β-catenin	Snail1 (Snail)
Smad-2/3	Snail2 (Slug)
NF-κβ	Twist
	β-catenin
Proteins whose activity increases	
ILK	Rho
GSK-3β	
***In-vitro* functional markers**	
Increased migration	Increased scattering
Increased invasion	Altered cell shape

EMT in Cancer

- Turning an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, adhesion and migration capacity.
- Thus there is a derangement of apicobasal polarity and cell-to-cell adhesive architecture and function, lack of basal lamina integrity.
- The occurrence of EMT during tumor progression allows tumor cells (i.e., ones that are noninvasive and nonmetastatic) to acquire the capacity to infiltrate surrounding tissue and ultimately metastasize to distant sites.
- The most compelling evidence for the involvement of EMT in oncogenesis is the ability of multiple EMT regulators to enhance tumor formation and/or metastasis.

Studies to Prove Role of EMT in Cancer

- For example, expression of Snail1 increases the aggressiveness of experimentally induced breast tumors, and high Snail1 expression correlates with an increased risk of tumor relapse and poor survival rates in human breast cancer.
- Loss of E-cadherin is a hallmark of metastatic carcinoma, and proteomic analysis of breast cancer reveals that circulating mammary tumor cells, or those found as micrometastases, show evidence of mesenchymal conversion.
- Nuclear localization of β-catenin is frequently used as an EMT marker, and nuclear β-catenin is a marker for a poor prognosis in colorectal cancer.

THERAPEUTIC IMPLICATIONS

- Strategies to block any steps during EMT would have a major impact on EMT and, thereby, on fibrosis, and cancer metastasis.
- Blockade of growth factor, cytokine and integrin pathways that lead to activation of signaling elements such as β-catenin and transcription factors Slug, Snail, Twist which assume critical roles in EMT may constitute potent strategies to counteract the progression from localized cancers to disseminated disease.

- EMT-associated molecules can be used as markers for the prediction of prognosis and response to targeted therapy.
- Commonly used molecular markers for EMT include increased expression of N-cadherin, vimentin, and integrins.
- IHC may be used to detect the expression of these molecular markers. IHC of EMT involved molecules may also be used to predict metastatic behavior.
- Nuclear localization of β-catenin is frequently used as an EMT marker, and nuclear β-catenin is a marker for a poor prognosis in colorectal cancer.
- Loss of E-cadherin is a hallmark of metastatic carcinoma.
- Blockade of EGFR signaling pathway by using anti-EGFR antibody or EGFR tyrosine kinase inhibitors such as gefitinib or erlotinib inhibit invasion.
- BMP-7 antagonizes TGF-β driven EMT in fibrotic kidney and heart inhibit disease development.

POINTS TO REMEMBER

- Accumulating evidence in recent years indicates that EMT is a critical process not only in development but also in tumorigenesis.
- Acquisition of EMT properties during tumor progression is associated with dissolution of epithelial integrity, increased migration, local invasion and, ultimately, metastasis.
- For its association with invasion and early steps of metastasis, inhibition of EMT appears a viable strategy for novel approaches to cancer therapy.
- However, given the complex, intertwined circuitry regulating EMT, effective deployment of anti-EMT therapeutics cannot be done without understanding the molecular alterations of the specific tumor being targeted.

BIBLIOGRAPHY

1. Basson MA. Signaling in cell differentiation and morphogenesis. Cold Spring Harbor Perspectives in Biology. 2012 Jun 1;4(6):a008151.
2. Giepmans BN, van IJzendoorn SC. Epithelial cell–cell junctions and plasma membrane domains. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2009 Apr 1;1788(4):820–31.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000 Jan 7;100(1):57–70.
4. Kim D, Xing T, Yang Z, Dudek R, Lu Q, Chen YH. Epithelial mesenchymal transition in embryonic development, tissue repair and cancer: a comprehensive overview. Journal of Clinical Medicine. 2017 Dec 22;7(1):1.
5. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. Nature Reviews Molecular Cell Biology. 2014 Mar;15(3):178.
6. Larue L, Bellacosa A. Epithelial–mesenchymal transition in development and cancer: role of phosphatidylinositol 3′ kinase/AKT pathways. Oncogene. 2005 Nov;24(50):7443.
7. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial–mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol. 2006 Mar 27;172(7):973–81.
8. Maru Y. Tumor Microenvironment. Inflammation and Metastasis 2016 (pp. 233–303). Springer, Tokyo.
9. Thiery JP. Epithelial–mesenchymal transitions in tumour progression. Nature Reviews Cancer. 2002 Jun;2(6):442.
10. Vinay K, Abbas AK, Fauston N. Robbins and Cotran Pathologic Basis of Disease. Saunders, Elsevier, China. 2005;8:208–21.

2 Dendritic Cell Migration in Health and Disease

INTRODUCTION

- Dendritic cells (DCs) were discovered by Ralph Steinman in 1973.
- They are a component of the innate immune system.
- Their main function is to process antigen material and present it on the surface to T cells, thus functioning as antigen-presenting cells.

Origin of Dendritic Cells

Location

They are seen in the following locations:

1. Skin: Langerhans cells in the epidermis, dermal dendritic cells.
2. Under epithelia of lungs, stomach and intestine.
3. Immature state in the blood.

Functions of Dendritic Cells

- Antigen presentation and activation of T cells.
- Induction and maintenance of immune tolerance.
- Maintaining immune memory in tandem with B cells.

Subsets of Dendritic Cells

- There are three main subsets as follows:
  1. Plasmacytoid dendritic cell (pDC)
  2. Conventional DC1 (cDC1)
  3. Conventional DC2 (cDC2)
- Minor subsets include—Monocyte-derived DC (MoDC) and double negative DCs.
- The immunological profile of cDC1 and cDC2 varies according to the various sites.

DC Migration Routes

Maturation and Mobilization of Tissue Resident DCs

- During embryonic development, as well as postnatally, DC progenitors populate the skin, mucosal surfaces and most solid organs of the body to give rise to immature classical dendritic cells (cDCs).

- These act as sentinels of the immune system.
- They display characteristic dynamic stellate-like extensions.
- These extensions allow these cells to exhibit sweeping movements and to engage in multiple contacts with each other.
- This leads to the formation of a dense cellular network for the efficient detection of invading pathogens.
- Immature cDCs are rather immotile and are specialized in the sampling of foreign as well as self-antigens.
- They use numerous mechanisms of antigen acquisition, including receptor-dependent endocytosis and macropinocytosis.
- Subsequently, they undergo activation.
- This is either triggered by an intrinsic program or due to the recognition of molecules associated with pathogens and microbiota.
- Subsequently an increase in motility corresponding to upregulation of CC-chemokine receptor 7 (CCR7) is seen.
- CCR7 interacts with its ligands CC-chemokine ligand 19 (CCL19) and CC-chemokine ligand 21 (CCL21).
- They are found on lymphatic endothelial cells.
- They guide DCs towards and into the lumen of initial lymphatic vessels (also known as 'terminal lymphatics').
- This process is known as haptotaxis.
- Haptotaxis is defined as persistent directional migration along gradients of a chemoattractant that are immobilized on cells and/or elements of the extracellular matrix.
- The terminal blind-ended lymphatic sacs are equipped with a discontinuous basement membrane and flap valves.
- These allow DC entry without pericellular proteolysis.

Intranodal Migration and Positioning of Dendritic Cells

- Once inside the afferent lymphatic vessel, DCs first crawl along the vessel wall but are then passively transported by the lymph flow into the subcapsular sinus (SCS) of lymph nodes.
- Subsequently they enter the lymph node parenchyma through the SCS floor.
- Atypical chemokine receptor 4 (ACKR4) is specifically expressed by lymphatic endothelial cells (LECs) lining the ceiling, but not the floor.
- This causes a CCL21 chemokine gradient that points towards the lymph node paracortex.
- This supports the efficient translocation of lymph-derived DCs into the T cell rich area.
- After their entry from afferent lymph, migratory DCs join the network of the largely sessile lymph node-resident DCs.
- These are embedded in the stromal network of the T cell areas and acquire antigen from the so-called lymph node conduits.
- Pre-cDCs engage L-selectin to gain access to lymph nodes via high endothelial venules (HEVs).

- Plasmacytoid dendritic cells (pDCs) also primarily enter lymph nodes via HEV.
- CCR7 again is responsible.

DC Migration in the Skin

DC Subsets in the Skin

- Langerhans cells (LCs), expressing C-type lectin langerin (CD207) are the only tissue resident DC population in the epidermis.
- Dermal DCs comprise several subsets including XC-chemokine receptor 1 (XCR1)+ conventional DC1s (cDC1s), CD11b+ conventional DC2s (cDC2s), and XCR1-CD11b- double negative cDCs
- In addition, and especially during inflammation, CCR2+ monocyte-derived DCs (moDCs) are recruited to the dermis.
- Like cDC2s, moDCs express CD11b.

LCs in the Epidermis

- LCs arise from yolk sac-derived erythromyeloid progenitors (EMPs).
- These cells populate the fetal liver and give rise to an initial wave essentially all tissue resident macrophages as well as skin LCs.
- These cells regenerate from local precursors throughout adult life.
- Transforming growth factor-β1 (TGF-β1) from both autocrine and paracrine sources as well as keratinocyte-derived interleukin 34 (IL-34) are required for the maintenance and homeostatic self-replenishment of LCs.
- In the noninflamed skin, LCs are nonmotile and 'anchored' to keratinocytes by E-cadherin-containing tight junctions.
- Also, TGF-β1 inhibits steady-state and inflammation-induced motility of LCs.
- Following activation in the skin, LCs reach the draining lymph node later than dermal DCs and populate different areas.
- LCs migrate into the deep T cell zone, dermal DCs reside at interfollicular areas and colonize regions directly beneath the B cell follicles.
- Recent evidence suggests that LCs promote *peripheral tolerance induction*.
- By migrating to cutaneous lymph nodes, LCs mediate deletional tolerance in CD4+ as well as CD8+ T cells, induce regulatory T (Treg) cell development and promote the production of immunosuppressive IL-10
- Immigration of (bone marrow-derived) blood-borne cDC precursors (pre-cDCs), plasmacytoid DCs (pDCs), monocytes and circulating (immature) cDCs into different mucosal and non-mucosal peripheral tissues via resting or inflamed postcapillary venules.

Dermal cDCs

- All dermal cDCs are derived from hematopoietic stem cell (HSC) derived progenitor cDCs (precDCs).
- These cells continuously repopulate the adult dermis.
- Recent evidence suggests that different precDC subsets exist in the adult bone marrow and blood that are already committed to the cDC1 or cDC2 lineage.
- Dermal cDC1s efficiently cross-present antigen.

- Dermal cDC1s carry antigens derived from the skin commensal *Staphylococcus epidermidis* to cutaneous lymph nodes and induce *S. epidermidis* specific IL-17-producing CD8+ T cells that home back to the skin.
- There, these commensal-specific T cells get further activated by IL-1 that is secreted by CD11b+ skin resident DCs (either cDC2s or moDCs).
- T cells generated by this 'division of labor' between different subsets of skin DCs are thought to contribute to antipathogen defense of keratinocytes in an IL-17 dependent manner.
- cDC2s can respond to thymic stromal lymphopoietin (TSLP) expressed by keratinocytes.
- It is strongly elevated in skin lesions of patients with atopic dermatitis.

MIGRATORY DCs OF THE INTESTINES

- Migration of intestinal DCs has an important role in the following
  - Immune surveillance and homeostasis of the gut
  - Maintenance of tolerance towards commensals and food proteins.
- Migratory intestinal DCs can be derived from three different sites:
  1. Lamina propria
  2. Peyer's patches
  3. Solitary intestinal lymphoid tissues (SILT).

Lamina Propria DCs

- Lamina propria contains (at least) three distinct populations of cDCs
  - Intestinal cDC1s: Characterized by expression of CD103 and lack of CD11b.
  - Intestinal cDC2s: CD103+CD11b+ .
  - Double negative DCs: CD103–CD11b-.
- Lamina propria-derived CD103+ cDCs in general, exhibit a special propensity to induce regulatory T (T-reg) cells.

Antigen Uptake in the Gut

- CD103+CD11b+ intestinal DCs are recruited from the lamina propria into the epithelial cell layer.
- These intraepithelial DCs are the first to acquire any antigen.

Intestinal pDCs Mobilize cDCs

- The pDCs themselves do not migrate to the draining mesenteric lymph nodes.
- However, they do mobilize lamina propria cDCs to migrate to mesenteric lymph nodes in a TLR- and tumor necrosis factor (TNF)-dependent manner.

DC Migration and Immune Responses to Commensals

- Intestinal DCs carry live commensals to the mesenteric lymph nodes.
- This triggers the production of secretory IgA, which subsequently helps to prevent mucosal penetration by commensals.
- Sampling of commensals by DCs drives mucosal TH17 cell differentiation.
- This occurs locally in the gut and does not rely on the migration of antigen-presenting cells to secondary lymphoid organs.

DC Migration During Intestinal Infection

- Acute intestinal infection can lead to tissue damage including leakage of lymph vessels.
- Accumulation of migratory DCs occurs in the surrounding mesenteric adipose tissue.
- If this DCs migration to the mesenteric lymph nodes is prevented, it results in compromised mucosal immune responses.
- This is the likely reason why persistent infections, gut inflammation, and environmental enteropathy cause weak performance of oral vaccines.

MIGRATION OF LUNG-DERIVED DCs

- Lung DCs migration to mediastinal lymph nodes depends on CCR7 and complement-mediated signaling.
- FTY720 and prostaglandin D2 suppress respiratory DC migration to mediastinal lymph nodes.
- Age-dependent increases in prostaglandin D2 levels result in impaired DC migration and reduced T cell responses.
- This is linked to poorer clinical outcomes in older patients with severe respiratory viral infections.

DC Migration and Asthma

- Inflammation during allergen challenge did not significantly alter DC motility.
- However, it was associated with accumulation of CD11b+ DCs near airways.
- This depends on CCR6 expression on DCs and fibroblast expression of the integrin $\alpha v\beta 8$ (a critical activator of TGFβ).
- $\alpha v\beta 8$-mediated TGFβ activation is known to enhance IL-1β-dependent fibroblast expression of the CCR6 ligand, CCL20.

DC Migration During Respiratory Infection

- In respiratory virus infection, migration of lung DCs to mediastinal lymph nodes is required for the generation of cytotoxic T cell responses.

Infection by Influenza Virus

- In the early post-infection phase CD103+ cDCs are the predominant lung DC population transporting influenza virus to the mediastinal lymph nodes.
- At a later stage CD11b+ DCs accumulate in the mediastinal lymph nodes and dominate CD8+ T cell stimulation.

Mycobacterium Tuberculosis

- Migration of lung DCs to mediastinal lymph nodes after *Mycobacterium tuberculosis* exposure requires IL-12p40.
- Gene ASAP1, encoding a regulator of DC migration, is associated with susceptibility to tuberculosis.
- Genetically determined excessive reduction of ASAP1 expression impairs migration of *M. tuberculosis*-infected DCs and may delay the onset of adaptive immunity.

DC MIGRATION IN THE CENTRAL NERVOUS SYSTEM (CNS)

- Cerebrospinal fluid has a role in excretion, physical protection and immunological function, allowing for the transport of CNS-derived soluble antigens and immune cells.
- CNS parenchyma is largely devoid of DCs under steady-state conditions.
- DCs primarily reside within the choroid plexus and the meninges of the non-injured, steady-state CNS.
- Macrophages reside in various CNS compartments under steady-state conditions, including:
  - Parenchymal microglia
  - Perivascular
  - Meningeal
  - Plexus macrophages.
- During CNS inflammation, there is an additional increase in monocyte-derived macrophages.
- Macrophages are thought to be rather non-migratory, primarily fulfilling *in situ* functions such as tissue homeostasis or local lymphocyte stimulation.

DC MIGRATION IN INFLAMMATORY DISEASES

Rheumatoid Arthritis

- Both immature and activated DC populations are found within the inflamed joint synovium in rheumatoid arthritis.
- Pannus (Representing ectopic lymphoid tissues) developing in rheumatoid arthritis allows for the entry of circulating pre-cDCs and/or cDCs from the blood by means of chemokine signals.
- These include CX3CR1, CCR9, CXCR4.

Systemic Lupus Erythematosus

- pDCs play a crucial role in systemic lupus erythematosus (SLE), especially in the early phases of the disease.
- Number of circulating pDCs within the blood of SLE patients is markedly reduced, and this correlates with disease activity.
- Thus, it is quite plausible that the recruitment of pDCs into lymph nodes and/or into diseased tissues, such as the skin, might contribute to SLE progression.
- SLE serum factors, in particular, high levels of type I interferon could synergize with bacterial lipopolysaccharide.
- They induce the differentiation of a subset of monocytes into CCR7-expressing migratory moDCs.
- This could explain the commonly observed phenomenon of SLE 'flares' triggered by infections.

Psoriasis

- Psoriasis is an inflammatory disorder of the skin, characterized by a massive infiltration of effector cells including the following:
  - Natural killer (NK) cells
  - Type 3 innate lymphoid cells (ILC3s)
  - TH1 cells
  - TH17 cells and γδ T cells, that drive the inflammatory process by producing IL-17 and IL-22.
- Keratinocytes themselves, produce IL-36 that stimulates dermal cDCs to produce IL-23, which in turn drives TH17 cell activation.

General Concepts of DC Migration

- Any antigen from the peripheral tissues can reach to lymph nodes in two ways:
  1. Using DCs
  2. As free soluble Ag
- The first method has certain distinct advantages. These are as follows:
  - Delivery of an adequate amount of antigen to lymph nodes is ensured. This leads to the induction of an appropriate immune response. For example, intracellular or tightly cell-bound antigens.
  - Retention of arriving DCs in draining lymph nodes helps to effectively 'concentrate' rare antigens within the lymph nodes, thereby enhancing antigen presentation to T cells.
  - Compartmentalization of immunity facilitates tissue-specific responses. For example, immune responses towards commensals are restricted to mucosal immune compartments and do not induce potentially detrimental systemic inflammatory reactions.

POINTS TO REMEMBER

- The migration from non-lymphoid to lymphoid tissue is a key feature of DCs.
- CCR7 is key in facilitating this process in generally all tissues.
- In steady-state conditions DC migration is essential for the induction of peripheral tolerance.
- The relative importance of migratory DCs for mounting protective immunity during infections seems to depend on both the pathogen itself and the tissue site affected.
- Mechanisms that allow precDCs to populate nonlymphoid organs during development and replenishment are not understood.
- Therapeutic approaches that target the migratory machinery of DCs possess an enormous potential for interfering with unwanted immune responses.
- In this regard strategies that target CCR7 or other key mediators of DC trafficking may represent promising novel approaches to target DCs in the clinic.

BIBLIOGRAPHY

1. Braun A, et al. Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. Nat. Immunol. 2011; 12: 879–87.
2. Diehl GE, et al. Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX3CR1hi cells. Nature 2013; 494: 116–120.
3. Farache J, et al. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity 2013; 38: 581–95.
4. McDole JR, et al. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature 2012; 483: 345–9.
5. Murphy TL, et al. Transcriptional control of dendritic cell development. Annu. Rev. Immunol. 2016; 34: 93–119.
6. Niess JH, Reinecker HC. Lamina propria dendritic cells in the physiology and pathology of the gastrointestinal tract. Curr. Opin. Gastroenterol. 2005; 21: 687–91.
7. Pascale F, et al. Plasmacytoid dendritic cells migrate in afferent skin lymph. J. Immunol. 2008; 180: 5963–72.
8. Shklovskaya E, et al. Langerhans cells are precommitted to immune tolerance induction. Proc. Natl. Acad. Sci. USA 2011; 108: 18049–54.
9. Steinman RM, Decisions about dendritic cells: past, present, and future. Annu. Rev. Immunol. 2012; 30: 1–22.
10. Worbs T, et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. J. Exp. Med. 2006; 203: 519–27.

3 Immune Reconstitution Inflammatory Syndrome

Anam Singh

INTRODUCTION

- Immune reconstitution inflammatory syndrome (IRIS) is a paradoxical clinical worsening of a known condition or the appearance of a new condition after initiating antiretroviral therapy (ART) in HIV-infected patients.
- Since clinical deterioration occurs during immune recovery, this phenomenon has been also described as immune restoration disease (IRD)/immune reconstitution syndrome (IRS)/paradoxical reactions.
- Antiretroviral therapy in HIV/AIDS patients leads to dramatic reductions in plasma viral load, improvement in CD4+ T cell counts and partial restoration of overall immune function.
- These immunological changes correlate with reduction in the frequency of opportunistic infections (OI) and prolonged survival.

HISTORY

- This condition was first noted following the introduction of Zidovudine monotherapy in the early 1990s.
- In these cases localized forms of *Mycobacterium avium*-intracellulare (MAI) infection were observed in association with the recovery rather than failure of cellular immune responses.

Epidemiology

- Despite numerous descriptions of the infectious and non-infectious causes of IRIS, the overall incidence of the syndrome itself remains largely unknown.
- However, it is estimated that 17–23% of patients initiating ART will develop the syndrome.
- The variation in reported frequency reflect differences in case definitions, and more importantly, differences in study populations with differing risk profiles and underlying burden of opportunistic infections.
- Most of the literature on epidemiology comes from the developed countries.
- In a series from southern India TB-IRIS was reported in 7.6% of patients receiving ART.

- It is expected that IRIS will become more common in resource-constrained settings like India, where access to ART is increasing.
- The underlying prevalence of opportunistic infections (OI) like *M. tuberculosis* is high in this setting and patients initiating ART are more likely to have advanced immune-suppression.
- Although IRIS is now a well-established entity, uncertainty exists with regards to its pathogenesis and management.
- Research in the field is hampered by lack of a consistent definition of the syndrome.

Risk Factors

- Male sex and younger age.
- Lower CD4+ cell count at ART initiation (especially <50 cells/μl).
- Higher HIV RNA at ART initiation.
- Lower CD4:CD8 ratio at ART initiation.
- More rapid initial fall in HIV RNA on ART.
- Shorter interval between OI therapy initiation and ART initiation.
- Disseminated versus localized OI.
- ART-naïve patient.

Diagnostic Criteria

- General IRIS case definition was proposed by ***French et al*** (2004).
- Diagnosis requires two major criteria or one major criterion plus two minor criteria to be fulfilled.

Major Criteria

1. Atypical presentation of opportunistic infections or tumors in patients responding to ART, manifested by any of the following:
   - Localized disease.
   - Exaggerated inflammatory reaction.
   - Atypical inflammatory response in affected tissues.
   - Progression of organ dysfunction or enlargement of pre-existing lesions after definite clinical improvement with pathogen-specific therapy prior to ART and exclusion of treatment toxicity and new diagnoses.
2. Decrease in plasma HIV RNA level >1 log10 copies/mL.

Minor Criteria

1. Increased blood CD4+ T cell count after ART.
2. Increase in immune response specific to the relevant pathogen, e.g. delayed hypersensitivity response to mycobacterial antigens.
3. Spontaneous resolution of disease without specific antimicrobial therapy or tumor chemotherapy with continuation of ART.
4. ***Robertson et al (2006)*** also gave their criteria. These are divided into required and supportive criteria.

Required criteria

- Worsening symptoms of inflammation/infection.
- Temporal relationship with starting of antiretroviral treatment (3 months).

- Symptoms not explained by newly acquired infection or disease or the usual course of a previously acquired disease.
- >1 log10 decrease in plasma HIV load.

Supportive criteria

- Increase in CD4+ cell count of ≥25 cells/μl.
- Biopsy demonstrating well-formed granulomatous inflammation or unusually exuberant inflammatory response.

Pathogenesis

- The immunopathology of IRIS is determined by the inciting pathogen.
- Both CD4+ and CD8+ effector T cells are involved in the pathogenesis.
- The syndrome appears to result due to an unbalanced immune reconstitution of effector (Pro-inflammatory Th17) and regulatory T cells in patients receiving ART.
- Macrophages and natural killer cells are also suspected to play a role in IRIS.
- Due to inappropriate activation of macrophages TB-IRIS develops.
- NK cells are responsible for herpes IRIS.
- Whether elicited by an infectious or non-infectious agent, the presence of an antigenic stimulus for development of the syndrome appears necessary.

Types of IRIS

IRIS is of 2 types:

1. Unmasking IRIS
2. Paradoxical IRIS

Unmasking IRIS

- It is defined as immune response against a pathogen that was not causing overt clinical disease before initiation of ART.
- This type of IRIS is characterized by atypical exuberant inflammation and/or an accelerated clinical presentation suggesting a restoration of antigen-specific immunity.
- When infectious it is the antigenic response to pathogenic infection, and when non-infectious it is due to antigenic response to self-antigen.
- This type of IRIS usually presents during the first 3 months of therapy.
- Viable pathogens may be isolated from samples obtained from affected body sites.
- Diagnostic criteria.
- Clinical criteria:
  - Temporal relationship: ART initiation must precede clinical deterioration.
  - New onset of symptoms of an infectious or inflammatory condition after initiation of ART.
  - Consistent with the presence of pre-existing causative pathogen or antigen at the time of starting ART.
  - Either of the following:
    - Onset within 3 months after initiating ART.
    - Atypical or exaggerated clinical, histological or radiological findings in terms of severity, character of inflammatory response, rapidity of onset or localization.

- Exclusion of other causes:
  - Event not explained by:
    - Expected clinical course of another condition.
    - Drug toxicity.
    - Newly acquired infection, based on clinical or other evidence.
    - Failure of antiretroviral therapy: Presumptive based on nonadherence or resistance to ART, or confirmed based on VL assay, if available.

Paradoxical IRIS

- It occurs when the opportunistic infection is present at initiation of ART which worsens on therapy.
- This may be a response to living pathogens or a response to the antigens of non-viable pathogens.
- This form is also most common during the first 3 months of therapy but may also present later**.**
- IRIS is precipitated by the degree of immune restoration following ART.
- An alternative immunological mechanism may involve qualitative changes in lymphocyte function or lymphocyte phenotypic expression.
- Following ART, there is restoration of immune response with redistribution of T cells from peripheral lymphoid tissue, causing increased memory CD4 cells.
- After this redistribution, naïve T cells increase and are thought to be responsible for the later quantitative increase in CD4+ cell counts.
- Thus, IRIS may be due to a combination of both quantitative restoration of immunity as well as alterations in qualitative function and phenotypic expression observed soon after the initiation of ART.
- Clinical criteria
  - Temporal relationship: ART initiation must precede clinical deterioration.
  - One of the following:
    - Worsening of an infectious or inflammatory condition that was recognized and ongoing at the time of ART initiation, following an appropriate response to clinical treatment.
    - Deterioration with atypical or exaggerated clinical, histological or radiological findings in terms of severity, character of inflammatory response, rapidity of onset or localization.
    - Recurrence of an episodic infectious or inflammatory condition, worse than episodes within 1 year preceding ART in terms of frequency, severity or response to therapy.
- Exclusion of other causes
  - Worsening not explained by:
    - Expected clinical course of underlying condition, given current therapy with the susceptibility profile of the organism.
    - Drug toxicity.
    - Other infection or inflammatory condition.
    - Withdrawal of previous effective therapy.
    - Failure of antiretroviral therapy: Presumptive based on nonadherence or resistance to ART, or confirmed based on VL assay, if available.

Biomarkers of IRIS

- Various inflammatory markers, chemokines and cytokines that signify innate and adaptive immune activation may act as useful biomarkers.
- The role of these biomarkers has been assessed mainly in TB-IRIS and cryptococcal meningitis (CM)-IRIS.
- These biomarkers include:
  - C-reactive protein (CRP)
  - Interferon (INF)-γ
  - Interleukin (IL)-2, 6, 7, 12, 13, 17, 18
  - Tumor necrosis factor (TNF)-α
  - INF-γ inducible protein-10 (IP-10)
  - D-dimer.

DISEASE SPECIFIC-IRIS

Mycobacterium tuberculosis IRIS

- *Mycobacterium tuberculosis* (TB) is among the most frequently reported pathogen associated with IRIS.
- Incidence varies from 8 to 43%.
- The incidence of IRIS is expected to rise in this patient group because of the wide availability of HAART in India now.

Risk Factors

- These include the following:
  - Paradoxical TB-IRIS
  - Shorter delay between commencing ATT and ART
  - Low baseline CD4+ cell count
  - Higher baseline viral load
  - Rapid reduction in viral load
  - Disseminated tuberculosis
- Majority of the cases develop IRIS within the initial 2 months of ART when the antigen burden is high.

Pathogenesis

- Recirculation of previously sequestered CD45RO memory lymphocytes allows the pathogen-specific cells to gain access to the sites of infection and mount an inflammatory response.
- In normal condition, when Mycobacterium infection acts on resting macrophage, CD4 cells produce IFN gamma to activate the macrophage to achieve bacterial containment.
- In case of HIV, there are a few or no CD4 cells and when Mycobacterium infection acts on resting macrophage, then it leads to formation of primed macrophages which get accumulated.
- When the CD4 cells are restored post-ART, accumulated primed macrophages produce increased inflammatory cytokines causing excessive inflammation and tissue destruction.

Case Definition for Tuberculosis-associated IRIS

- This was given by ***Colebunders et al***, 2006.
- **Suspected case** (all 3 should be met)
  - An initial clinical response to ATT.
  - New persistent fevers without another identifiable cause and/or one or more of the following:
    - Worsening or emergence of dyspnea
    - Stridor
    - Increase in lymph node size, development of abscesses
    - Development of abdominal pain with ultrasound evidence of abdominal lymphadenopathies
    - Unexplained CNS symptoms.
  - Adequate adherence to ART and tuberculosis treatment
- **Confirmed case** (must meet the following three criteria)
  - New/worsening radiological signs.
  - A good virological response and/or increase in CD4+ lymphocyte count, and/or conversion of tuberculin skin test from negative to positive
  - Adequate adherence to ART and ATT.
  - Exclusion of treatment failure or other concomitant infections, tumors, or allergic reactions.

Treatment

- Treatment for mycobacterial-associated IRIS depends on the presentation and disease severity.
- Most patients present with non-life-threatening presentations which respond to the institution of appropriate ATT.
- Treatment can also be done with NSAIDs and severe cases with corticosteroids.

Atypical Mycobacteria

- Atypical mycobacteria are also frequently reported as causative pathogens in IRIS.
- MAC-associated IRIS typically develops in severely immunosuppressed individuals, who have an excellent response to ART.
- The syndrome manifests usually after 2–8 weeks of ART in patients with low CD4 counts.

Clinical Features

- Fever
- Suppurative painful lymphadenitis
- Followed by pulmonary disease
- May also involve joints, spine, skin and soft tissue
- In contrast to disseminated MAC disease of advanced AIDS, MAC IRIS usually presents as localized disease.
- Treatment is similar to TB-IRIS.
- Occasionally, surgical excision of profoundly enlarged nodes or debridement of necrotic areas has been reported.

Cytomegalovirus (CMV) Infection

- CMV retinitis may be seen either in patients with a prior history of CMV retinitis or in patients with no previous evidence of retinitis.
- It is speculated that an ART-induced inflammatory response may be responsible for unmasking a subclinical infection.
- This is seen exclusively in people with previous CMV retinitis infection who responded to ART therapy (paradoxical).
- In addition to classical CMV retinitis, ART leads to new clinical manifestations of the infection, termed immune recovery vitritis (IRV) and immune recovery uveitis (IRU).

Immune recovery vitritis

- IRV presents with acute onset of blurred vision and "floaters" caused by posterior segment inflammation.
- Ophthalmologic examination reveals numerous inflammatory cells in the vitreous humor.
- Symptoms usually resolve in one month without specific treatment and without any lasting visual effects.

Immune recovery uveitis

- IRU may occur within months of ART initiation, but typically is a late complication; occurring about 3 years after patients begin ART.
- It often results in macular edema, epiretinal membrane formation, and/or cataracts, which can lead to permanent vision loss and hence requires a high index of suspicion.

Treatment

- Anti-CMV therapy with gancyclovir or valgancyclovir
- However, IRU may not respond to anti-CMV therapy
- The use of systemic corticosteroids has been successful, and IRV may require peri-ocular corticosteroid injections.

Varicella Zoster Virus Infection

- With the introduction of protease inhibitors (PI), increasing rates of herpes zoster were noted in HIV-infected patients.
- Incidence rates are three to five times higher than those observed in the pre-HAART era.
- Mean onset of disease from ART initiation is 5 weeks (range 1–17 weeks).

Pathogenesis

- Normally, CD8 cells are implicated in the reactivation of varicella zoster infection in non-HIV patients.
- Therapy with PI causes an increase in CD4 as well as CD8 cells.
- Most common presentation is typical dermatomal involvement without dissemination or systemic symptoms.
- Although complications such as encephalitis, myelitis, cranial and peripheral nerve palsies, and acute retinal necrosis can also occur.

Cryptococcus Neoformans (CN) IRIS

- Paradoxical CN-IRIS incidence has been reported to be 10–42%, in ART naïve patients with *Cryptococcus.*
- Approximately 60% of cases occur within the first month, although the symptoms may present as late as 10 to 12 months after treatment.

Clinical Features

- Meningitis, lymphadenitis, pneumonitis, and localized abscess.
- Overall incidence of neurological IRIS is 1.5% in individuals with CD4+ counts <200 cells/μl.
- The majority of cryptococcal IRIS cases represent reactivation of previously treated cases, suggesting either an immunological reaction to incompletely treated disease or an inflammatory reaction to residual antigens.
- Unlike many other forms of IRIS, which produce less dramatic consequences, CM-IRIS is exceptional for its substantial morbidity and mortality.

Treatment

It has three phases:

1. Induction phase for 14 days with amphotericin B
2. Consolidation phase with fluconazole for 8 weeks
3. Suppressive phase with maintenance dose of fluconazole.

Pneumocystis jiroveci Pneumonia

Immune reconstitution inflammatory syndrome may present as worsening pulmonary symptoms and high fever in patients who had been improving on PCP therapy or in patients with recent successful treatment of PCP.

IRIS AND MALIGNANCY

Kaposi Sarcoma

- Kaposi sarcoma (KS) is a multifocal systemic tumor with origin in the endothelial cells which was first described by Moriz Kaposi in 1987.
- It is classified into four variants:
  1. Classic KS
  2. Endemic or African KS
  3. Iatrogenic KS
  4. AIDS-associated KS
- The AIDS-KS is the most prevalent form at present. Its first line therapy is ART.
- ***French et al*** defined IRIS-associated KS as:
  - An abrupt clinical worsening of a previously existing KS (paradoxical) or a new presentation of a previously unknown KS (unmasking) in temporal association with initiation or re-initiation of ART or change to a more active regimen
  - Concomitant reduction of at least 1 $\log_{10}$ in the HIV-1 RNA levels at the time of the IRIS event or with two of the following three minor criteria:
    1. Twofold increase in CD4+ T cell count after ART.
    2. Increase in the immune response (HHV-8 antibodies).
    3. Spontaneous resolution of disease without specific chemotherapy with continuation of ART.

Pathogenesis

- The synergy between the humoral and cellular immune response against HHV8 secondary to ART results in inflammation and tumorigenesis characterizing the clinical presentation of IRIS-KS.
- Most AIDS-KS patients have undetectable or very low HHV8-specific cytotoxic T lymphocytes.
- After the initiation of ART, CD8 T specific for HHV8 are detectable, and the recovery of this cell population is thought to be partly responsible for IRIS-KS.
- After ART therapy, there is an increase in the lymphocytes producing TNF-α, INF-γ and IL-1β which causes a reactivation of latent HHV8 and up-regulation of integrins like **MMP and VEGF.**
- The TAT protein encoded by the HIV virus binds to the Integrin receptors on the KS cells, thereby promoting mitogenic stimulus to VEGF.

Treatment

- Treatment with corticosteroids is not recommended because glucocorticoid treatment promotes HHV8 replication and tumor growth, worsening disease progression.
- The regimen with HAART and chemotherapy demonstrate an effective suppression of HHV8 viral replication.

Non-Hodgkin's Lymphoma

- Non-Hodgkin's lymphoma (NHL) have also been reported as a rare manifestation of immune reconstitution, however, literature is scant regarding lymphomas.

POINTS TO REMEMBER

- It is extremely important to be aware that ART-engendered immune recovery may result in pathological inflammation in a subset of patients.
- Vigilance needs to be especially high during the first several months of therapy when the incidence of IRIS peaks.
- However, cases continue to occur even after 1 or 2 years of therapy.
- More studies are needed to elucidate the pathogenesis and establish better treatment modalities.
- IRIS is a clinical entity requiring high level of suspicion, though not fatal, still requiring supportive care.
- Due to the lack of properly laid down diagnostic criteria, this entity is mostly underdiagnosed.

BIBLIOGRAPHY

1. Bosamiya SS. The immune reconstitution inflammatory syndrome. Indian J Dermatol. 2011;56(5):476–79.
2. French MA, Price P, Stone SF. Immune restoration disease after antiretroviral therapy. AIDS. 2004;18:1615–27.
3. Gopal R, Rapaka RR, Kolls JK. Immune reconstitution inflammatory syndrome associated with pulmonary pathogens. Eur Respir Rev. 2017;26: 160042.

4. Koh KC, Pak JW. Immune reconstitution inflammatory syndrome in a HIV-infected patient with disseminated tuberculosis. Malays Fam Physician. 2016;11: 27–30.
5. Murdoch DM, Venter WD, Van Rie A, Feldman C. Immune reconstitution inflammatory syndrome (IRIS): A review of common infectious manifestations and treatment options. AIDS Res Ther. 2007;4:9.
6. Robinson MR, Reed G, Csaky KG, Polis MA, Whitcup SM. Immune-recovery uveitis in patients with cytomegalovirus retinitis taking highly active antiretroviral therapy. Am J Opthalmol. 2000;130: 49–56.
7. Sanjay SB. The immune reconstitution inflammatory syndrome. Indian J Dermatol. 2011; 56: 476–9.
8. Shelburne SA, Visnegarwala F, Darcourt J, Graviss EA, Giordano TP, et al. Incidence and risk factors for immune reconstitution inflammatory syndrome during highly active antiretroviral therapy. AIDS. 2005;19: 399–406.
9. Surjushe AU, Jindal SR, Kamath RR, Saple DG. Immune reconstitution inflammatory syndrome. Indian J Dermatol Venereol Leprol. 2006;72:410–4.
10. Walker NF, Scriven J, Meintjes G, Wilkinson RJ. Immune reconstitution inflammatory syndrome in HIV-infected patients. HIV AIDS. 2015;7: 49–64.

4 Principles and Clinical Utility of Measuring Immunoglobulin Subclasses

Poorvi Kapoor

INTRODUCTION

- Immunoglobulin is a glycoprotein that is made in response to an antigen and can recognize and bind to the antigen that caused its production.
- They derive their name from the finding that they migrate to the region of globulins, when serum is placed in an electrical field.
- They are gamma globulins secreted from plasma cells.
- They constitute 25–30% of total serum proteins, and are present in serum, tissue fluids and mucosal surfaces.

BASIC STRUCTURE

- They are composed of two heavy and two light polypeptide chains, which are linked by disulphide bonds.
- These chains are subdivided into constant and variable regions, which contain an amino terminal in the variable region and carboxy terminal in the constant region.
- Light chains occur in two varieties, kappa and lambda and have a single variable (VL) and single constant domain (CL).
- Heavy chains, on the other hand, have a single variable domain (VH) and three constant domains (CH1, CH2, CH3) and the hinge region is seen between the CH1 and CH2 domains.
- On digestion with proteolytic enzymes (like papain), the peptide bonds in the hinge region are broken and produce three fragments.
- Two identical fragments, known as Fab, have antigen binding activity, and the other Fc fragment has effector functions and is responsible for complement fixation, opsonization and placental transfer.

Classification

They are classified into 5 types based on differences in the amino acid sequences of the constant portion of the heavy chains:

- IgG (gamma)
- IgA (alpha)
- IgM (mu)
- IgD (delta)
- IgE (epsilon).

Immunoglobulin G

- It is the most abundant class in the serum and constitutes 80% of total immunoglobulins.
- It contains less carbohydrates than other immunoglobulins and has a half-life of 23 days, which is the longest of all Ig subtypes.

Functions

- It crosses the placenta and provides immunity to the fetus and neonate at birth.
- It acts against bacteria and viruses by opsonization.
- Activates complement via the classical pathway.
- It has 4 subclasses which vary in the number of disulphide bonds and length of the hinge region: IgG1, IgG2, IgG3 and IgG4.
- IgG1, IgG3, IgG4 cross the placenta and protect the fetus.
- IgG1 and IgG3 bind Fc receptor on phagocytic cells and mediate opsonization.

Immunoglobulin A

- It is the second most common globulin and constitutes 10–15% of total immunoglobulins.
- It is present in milk, saliva, tears, mucosa of respiratory tract, digestive tract and genitourinary tract and is present as a monomer in serum, and a dimer in secretions.
- The J chain is associated with the dimeric form, has a half-life of 6–8 days and has two subclasses, IgA1 and IgA2
- The secretory piece is made in epithelial cells and is added to the IgA (made in plasma cells), as it passes into the secretions.
- This secretory piece helps IgA to be transported across mucosa and also protects it from degradation.

Functions

- It provides local immunity and provides an important line of defense against *S. typhi*, *V. cholerae*, *N. gonorrhoeaes* as well as influenza and polio virus.
- IgA secreted in breast milk protects the infant in the first few months of life.
- It activates complement via the alternate pathway and promotes phagocytosis and intracellular killing of organisms.

Immunoglobulin M

- It constitutes 5–10% of total serum proteins and is a polymer of five monomeric units (pentamer).
- It is held together by disulphide bonds and the J chain.
- IgM is mostly present in the intravascular compartment, has a half-life of 5 days and is the first immunoglobulin to be produced in primary response to an antigen.
- It is relatively short lived and therefore its demonstration in the serum indicates a recent infection.
- When detected in the serum of newborns, it indicates a congenital infection.

Functions

- It agglutinates bacteria and activates complements via the classical pathway.
- It is responsible for opsonization and is also responsible for immune-mediated hemolysis.

Immunoglobulin E

- Its structure is similar to IgG and it has a half-life of 2 days
- It is mostly present in extracellular location
- It is heat labile with a serum concentration of 0.3 μg/mL.

Functions

- It binds to the Fc receptor on the membranes of basophils and mast cells and mediates the release of histamine and other mediators of anaphylaxis
- It is responsible for anaphylactic ractions, hay fever, asthma and immediate hypersensitivity
- It also plays a role in immunity against helminths.

Immunoglobulin D

- Its structure is also similar to IgG and it constitutes 0.2% of total immunoglobulins
- It has a half-life of 3 days and is found in low levels in the serum
- Its role in the serum is uncertain, but it is primarily found on B cell surfaces where it functions as a receptor for antigens

Clinical Utility of Measuring Ig Subclasses

- The diagnosis of immunodeficiency is difficult, some of which include lack of screening, extensive use of antibiotics masking clinical presentations and lack of specific physical abnormalities associated with deficiencies.
- Immunodeficiency states should be suspected when there is:
  - ≥1 systemic bacterial infections (sepsis, meningitis, etc.)
  - ≥2 serious respiratory or documented bacterial infections like cellulitis, otitis media, pneumonia, lymphadenitis, etc. within 1 year
  - Unusual sites of infection like the brain or liver
  - Or unusual pathogens like *P. jiroveci*, *Aspergillus*, *S. marcescens*, *Nocardia* or *Burkholderia*
- The European Society of Immunodeficiency (ESID) has stated 10 warning signs of immunodeficiency.
  1. ≥8 new ear infections within 1 year
  2. ≥2 serious sinus infections within 1 year
  3. ≥2 months of antibiotic intake with little effect
  4. ≥2 pneumonias within 1 year
  5. Failure of an infant to gain weight or grow normally
  6. Recurrent deep skin or organ abscesses
  7. Persistent oral thrush or fungal infections of the skin
  8. Need for IV antibiotics to clear infections
  9. ≥2 deep seated infections
  10. A family history of primary immunodeficiency.
- Age of presentation varies depending on the cause of immunodeficiency:
  - Onset <6 months of age suggests a T cell defect
  - Between 6–12 months may help suggest a combined B and T cell defect
  - Presentation after 1 year of age suggests a defect in immunoglobulins

- Common defects can either have a known or unknown genetic basis
  - *Known genetic basis:*
    - Bruton agammaglobulinemia
    - Hyper-IgM syndrome
  - *Unknown genetic basis:*
    - Common variable immunodeficiency
    - Selective IgA deficiency
    - IgG subclass deficiency
    - Transient hypogammaglobulinemia of infancy.

Bruton's (X-Linked) Agammaglobulinemia

- It is due to mutation in Bruton tyrosine kinase and occurs in the later part of the first year of life.
- There are recurrent bacterial infections, usually with Pneumococci, Streptococci, Meningococci, *Pseudomonas* and *H. influenzae*.
- All the classes of immunoglobulins are grossly depleted and there is a marked decrease of B cells in circulation, with no formation of antibody even after injecting antigens.
- The tonsils and adenoids are atrophic but the thymus is normal.

Hyper-IgM Syndrome

- It can be X-linked which is associated with CD40 ligand defect (HIGM-1), with these cases associated with *P. carinii* and autoimmune disease.
- The autosomal recessive form is associated with mutation in gene cytidine deaminase (HIGM-2). There is lymphoid hyperplasia.
- Low IgG and IgA levels are seen with elevated IgM.
- There is hemolytic anemia with neutropenia and thrombocytopenia along with renal lesions.

Common Variable Immunodeficiency

- It occurs between 15–35 years of age.
- There is late onset hypogammaglobulinemia with recurrent pyogenic infection and an increased incidence of autoimmune disease.
- The total immunoglobulin levels are low with defective B cells in circulation.
- There is an increase in suppressor T cell with decreased helper T cell activity.

IgA Deficiency

- It can be inherited in an autosomal recessive or dominant pattern and occurs in 1 in 400 to 3000 individuals.
- There may be no presenting symptoms or they can present with sinopulmonary or gastrointestinal infections like giardiasis.
- It is characterized by serum IgA values < 10 mg/dL along with normal values of IgM and IgG.
- They may develop autoimmune diseases and can present with atopy or allergy to cow's milk.
- There is a risk of developing antibodies to IgA upon receiving blood products.

IgG Subclass Deficiency

- This deficiency is clinically very heterogeneous and co-exists with abnormal expression of > 1 subclass antibody, of which IgG2 or IgG3 deficiencies are the most common
- Clinically, they present with frequent bacterial infections of both upper and lower gastrointestinal tracts
- Since IgG1 is the most abundant subclass, a fall in the value of IgG1 causes total IgG to fall below normal, thereby causing hypogammaglobulinemia
- IgG4 levels are usually quite low in children, so a deficiency of this subclass presents later in life, usually after 10 years of age.

Transient Hypogammaglobulinemia of Infancy

- Maternally transmitted IgG gets catabolized by the 3rd or 4th month of life
- Symptoms occur due to slow maturation of the immune system which leads to a delay in immunoglobulin production
- Clinical presentation includes recurrent otitis media and respiratory tract infections
- Spontaneous recovery occurs between 18–30 months of age.

There are certain conditions which are associated with increase in immunoglobulin levels.

Conditions Associated with Increased IgE

Allergic Bronchopulmonary Aspergillosis

- An elevated level of serum IgE >416 IU/mL is one diagnostic criteria
- Levels of IgE are used to monitor the course of the disease and these levels fall with successful treatment with steroids and raise during disease exacerbation
- There is an association between disease activity and levels of Aspergillus antibodies

Asthma

- Atopic asthma is strongly related to increased levels of serum IgE
- A positive family history and skin test with the offending antigen induces an immediate wheal and flare response
- An anti-IgE monoclonal antibody, omalizumab, is an approved drug for use in asthmatics.

HyperIgE Syndrome

- It is an autosomal dominant disorder and is associated with a mutation of STAT3
- The autosomal recessive variant is associated with TYK2 and DOCK8 mutation
- These cases present with recurrent infections with *Staphylococcus aureus* and *Candida albicans*
- There are associated abnormalities like prominent forehead, hypertelorism, broad nasal bridge, facial asymmetry and hemihypertrophy
- IgE values are well above 2000 IU/mL and there is associated eosinophilia. They are also associated with an increased risk of lymphomas.

Conditions Associated with Increased IgM

Immunodeficiency Associated with HyperIgM

- It is an X-linked disorder associated with CD40 ligand defect (HIGM1) with an increased susceptibility to *P. carinii* and other autoimmune diseases.
- The autosomal recessive inheritance is associated with a mutation in cytidine deaminase (HIGM2) and is associated with lymphoid hyperplasia
- Elevated IgM levels are seen with low IgA and IgG levels.
- There can also be associated hemolytic anemia, neutropenia, thrombocytopenia and renal lesions.

Primary Investigations

- **Clinical history**
  - Recurrent and persistent infections
  - Infections due to unusual microbes
- **Physical examination**
  - Pale, lethargic, appearing chronically ill
  - On examination, there may be small or absent peripheral lymph nodes, tonsils or adenoids in infants and children
  - There may be petechiae and eczematous rashes over the skin
- **Complete blood count**
- **Radiological imaging**
  - X-rays show chronic interstitial infiltrates, absence of thymic shadow and bony abnormalities
  - CT scan may show pulmonary fibrosis and bronchiectasis, especially in CVID
- **Quantitative immunoglobulin assay**
  - Serum IgG, IgM and IgA levels
  - IgG subclass analysis.

Role of Immunoglobulins in Plasma Cell Disorder

- Plasma cell disorders are neoplastic proliferations of plasma cells with the production of immunoglobulins
- Various plasma cell disorders are as follows:
  - Multiple myeloma
  - Monoclonal gammopathy of undetermined significance
  - Asymptomatic multiple myeloma
  - Light chain multiple myeloma
  - Non-secretory myeloma
- Myeloma cells secrete IgG in 60% cases, less often IgA (20%), light chains (15%) and very rarely IgD, IgE and IgM
- It is important to evaluate the M component on electrophoresis for diagnosis
- Serum free light chain (SFLC) assay is useful for diagnosing, monitoring as well as for prognostication in these conditions
- This assay measures kappa and lambda ratio, with the normal ratio ranging between 0.26 to 1.65
- The diagnostic sensitivity of SFLC assay is highest for multiple myeloma (96.8%) and lowest for monoclonal gammopathy of undetermined significance (42%)

- Serum electrophoresis, nephelometry, radial immunodiffusion and immunofixation are the methods commonly used for SFLC assaying.

Nephelometry

- In this method, the light which is scattered from the antigen-antibody complex is measured
- **Principle:**
  - On the passage of an electromagnetic radiation (light), some amount of light is absorbed by the solution, and some is scattered or reflected
  - The scatter of this light is proportional to the concentration of insoluble particles present in the solution
  - When a sample containing an antigen is added to a cuvette containing the corresponding antisera, there is formation of an antigen-antibody complex
  - A laser diode then generates a beam of light, which is passed through this cuvette and the scatter is measured
  - A reference curve is generated from a standard containing known quantities of antigen
  - The scattered light can then be read from this curve
  - The intensity distribution of the scattered light depends on the relationship of the particle size of the generated complex to the wavelength of light used
- **Factors affecting nephelometry:**
  - Particle size and concentration (preferred for small particles in low concentration)
  - Distance of the light source
  - Wavelength of light used
- **Applications**
  - It is used primarily to measure the concentrations of immunoglobulins and serum proteins (IgG, IgA, Albumin, etc.)
  - It can also be used to monitor therapeutic drug levels in blood samples.

Radial Immunodiffusion

- It is a single diffusion technique used where the antibody is put into the gel and antigen is measured by the size of the precipitin ring formed when it diffuses out in all directions
- **Principle:** An antigen (in the sera of patients) is added to an antibody rich media and the antigen keeps reacting with the antibody till the zone of equivalence is reached. The area of ring is a measure of the concentration of the antigen present.

Serum Electrophoresis

- In this technique, the patient's serum is placed on a supporting medium made of cellulose or agar, which is then placed in an electrophoresis chamber and exposed to an electric current.
- The serum proteins get separated into 5 components: albumin, alpha 1 and alpha 2 globulin, beta and gamma globulins in the form of bands

Immunofixation

- It is used in serum or urine samples to identify the nature of M protein and is very sensitive.

- In this method, monospecific antibodies are used to identify specific types of immunoglobulin and light chains.
- The serum is placed on agarose gel in 5 different lanes and monoclonal antibody is then applied over the surface of the gel. The formed antigen–antibody complex is then visualized by staining.

Treatment

- Antimicrobial prophylaxis
- Immunization (all live vaccines avoided)
- Immunoglobulin therapy

POINTS TO REMEMBER

- Immunoglobulin is a glycoprotein that is made in response to an antigen and can recognize and bind to the antigen that caused its production.
- IgA deficiency is more common, followed closely by IgG.
- The ten warning signs of deficiency should be kept in mind and onset of these diseases usually occur after 1 year of age.
- Immunodeficiency diseases are difficult to diagnose, especially due to extensive use of antibiotics masking the classical presentation.
- These deficiencies require extensive clinical and laboratory tests to confirm the diagnosis.

BIBLIOGRAPHY

1. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, et al. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. Blood. 2009; 113(16):3716–25.
2. Elena E Perez, et al. Update on the use of immunoglobulin in human disease: A review of evidence. Work Group Report of the American Academy of Allergy, Asthma and Immunology. 2017; 1–46.
3. Glocker E. et al. Common variable immunodeficiency in children. Curr Opin Pediar. 2007;19:685–92.
4. Kassim AA, Neeiapu SS, Kwak LW. et al. Immunotherapy. In: Wintrobe's Clinical Hematology. 12th edn. Lee G, Bithell TC, Foerster J. et al (eds) . Lea and Febiger, Philadelphia. 2009;2:1721–46.
5. Moccia A, Ghielmini M. Monoclonal antibodies for the treatment of hematologic malignancies: schedule and maintenance therapy. Semin Hematol. 2008;45:75–84
6. Pan Q, Hammarstrom L. Molecular basis of IgG subclass deficiency. Immuno Rev. 2000; 178:99–110. doi:10.1034/j.1600–065X.2000.17815.
7. Pone EJ, Zan H, Zhang J, Al-Qahtani A, Xu Z, Casali P. Toll-like receptor and B cell receptors synergize to induce immunoglobulin class-switch DNA recombination: relevance to microbial antibody responses. Crit Rev Immunol. 2010; 30(1):1–29. doi:10.1615/CritRevImmunol.v30.i1.10
8. Pone EJ, Zhang J, Mai T, White CA, Li G, Sakakura JK, et al. BCR-signalling synergizes with TLR-signalling for induction of AID and immunoglobulin class-switching through the non-canonical NF-kappaB pathway. Nat Commun. 2012; 3:767. doi:10.1038/ncomms1769
9. Schur PH. IgG subclasses. A historical perspective. Monogr Allergy. 1988; 23:1–11.
10. Vlug A, Nieuwenhuys EJ, van Eijk RV, Geertzen HG, van Houte AJ. Nephelometric measurements of human IgG subclasses and their reference ranges. Ann Biol Clin (Paris). 1994; 52(7–8):561–7.

5 Microparticles

WHAT ARE MICROPARTICLES?

- First described in 1967 by Wolf as platelet membrane fragments in human plasma.
- Microparticles (MPs) are circulating phospholipid rich, submicron particles (0.05 –1.5 μm in size).
- They are released from the membranes of endothelial cells, platelets, leucocytes and erythrocytes.

Characteristics of Microparticles

- MPs are small particles.
- Their membranes consist mainly of lipids and proteins.
- They expose the anionic phospholipid phosphatidylserine.
- They also express membrane antigens that reflect their cellular origin and the cellular processes triggering their formation.
- Their shelf life is quite long (≈ 6 days).
- They are released from many blood cells.
- MPs bind to Annexin V, in the presence of Ca^{2++}.
- They carry CD markers, Tissue factor (TF), GP IIb-IIIa, etc.

Cellular Origin of Microparticles

- **Platelets** during activation of coagulation.
- **Endothelial cells** in auto-immune diseases, TTP and activation of coagulation.
- **Leucocytes** during the process of inflammation.
- **Erythrocytes** in cases of sickle cell anemia.

Generation of Microparticles

- Microparticles are released from the surface of cells following cell activation or apoptosis.
- The triggers include chemical stimuli, such as cytokines, thrombin and endotoxin, or physical stimuli, such as shear stress or hypoxia.
- Following cell activation, microparticle formation is dependent on a rise in the cytosolic calcium concentration with consequent activation of calpain and protein kinases and phosphatase inhibition.

- These changes result in cytoskeletal reorganization, membrane blebbing and the formation of microparticles.
- All these steps are shown in Fig. 5.1.

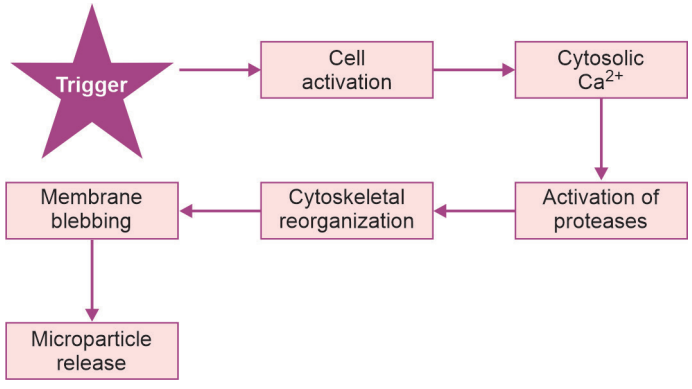

Fig. 5.1: Steps of generation of microparticles

Platelet Microparticles

- The platelet derived MPs are often seen at the site of blood vessel injury.
- These contribute to the process of hemostasis by providing a large surface for activation of the coagulation cascade leading to formation of the fibrin clot.
- MPs harbor active tissue factor (TF). Together they act synergistically as potent triggers of blood coagulation with TF-bearing MPs representing the so-called blood-borne TF.
- In contrast, pathologic MPs at high concentrations can predispose to thrombosis.
- Thus, they can be viewed as a major therapeutic target for arterial thrombosis, venous thrombosis and atherosclerosis.
- Apart from their procoagulant function MPs could also be involved in anticoagulant activity.
- It is due to the presence of tissue factor pathway inhibitor (TFPI) and antithrombin activity.

Endothelial Microparticles

- Microparticles have been shown to be released during apoptosis induced *in vitro* by growth factor deprivation or complement proteins.
- Both the cell of origin and the nature of the trigger influence the number and phenotype of the microparticles released and, consequently their pathophysiological effects.
- For example, endothelial microparticles (EMPs) released following apoptotic stimuli have higher levels of surface Annexin V binding to phosphatidylserine, and of constitutive endothelial cell markers such as CD31 (Platelet Endothelial Cell Adhesion Molecule, PECAM).
- EMPs induced by activation with TNF-α expressed higher levels of inducible antigens, such as CD62E (E-selectin), which were also increased on the parent endothelial cells.
- Total EMPs levels can be used as a surrogate marker of endothelial disturbance.
- The phenotypic profile of EMPs can help to discriminate between endothelial activation and apoptosis.

- For example, ratio of CD62+ (E-selectin) EMPs to CD31+ (PECAM) EMPs has been shown *in vitro* to be high in activation, low in apoptosis.
- Previously MPs were considered inert cell debris but now there is a growing evidence from *in vitro* and some *in vivo* studies that MPs may exercise a number of pathophysiologic functions.

MPs Functions

- Intercellular transfer of membrane proteins and lipids.
- Prothrombotic effects
- Proinflammatory effects
- Impairment of endothelial function
- Modulation of vascular tone
- Induction of angiogenesis and enhancement of cancer cells metastasis.

Role in Endothelial Function

- Endothelial microparticles have been shown to reflect endothelial activity, being released following activating or injurious external stimuli, and to themselves induce changes in endothelial function.
- Endothelial dysfunction is a common feature of many vascular disorders including atherosclerosis, diabetes, antiphospholipid syndrome, TTP and sickle cell disease.
- Endothelium-derived nitric oxide (NO) is the major mediator of acetylcholine-induced vasorelaxation of rat aorta *in vitro*.
- Exposure of rat aorta to EMPs obtained from cultured endothelial cells, resulted in impaired acetylcholine induced relaxation and reduced NO production.
- The same effect was also observed when using circulating microparticles obtained from patients following myocardial infarction (MI).
- Circulating EMPs may also contribute to the vascular changes seen in end stage renal disease through inhibition of the endothelial NO pathway.

Cellular Interactions

- Microparticles bear antigens of their cell of origin and can transfer these surface molecules to other cell types.
- The binding of microparticle surface antigens to their specific counter-receptor may induce intracellular signaling pathways and may alter the biological activity of the recipient cells.
- They are the consequence of the disease but can also be the cause of further clinical complications, by stimulating the blood procoagulant pathways.

Association of MPs with Diseases

- They have been reported to be associated with following diseases:
  - Hypertension
  - Diabetes
  - Myocardial infarction
  - Cancer
  - Acute coronary syndrome
  - Lupus anticoagulant

- Sepsis
- Pre-eclampsia.

- There is insufficient evidence to demonstrate that this association is causal.
- Definitely, elevated blood MPs in several disease processes result from inflammation, cellular activation and apoptosis.

Microparticles and Blood Transfusion

- In stored human blood, MPs are derived from platelets, leukocytes, and erythrocytes.
- Erythrocyte and platelet derived MPs have been implicated as mediators for:
  - Transfusion-related acute lung injury (TRALI)
  - Febrile nonhemolytic transfusion reactions
  - Thrombocytopenia
  - Urticaria
  - Thrombosis.

MICROPARTICLES: AS DIAGNOSTIC MARKERS

- Elevated in myocardial infarction (two to ten times)
- Increased in stroke.
- Increased levels predict vascular complications in diabetes
- Elevated levels have been also observed in certain cancers, for example, carcinoma breast.
- MPs levels correlate well with severity of hypertension.
- MPs are useful in follow-up and therapeutic monitoring of patients with myocardial infarction.
- Up to ten times elevation has been reported in hemophilia.
- MPs derived from PLTs, RBCs, WBCs, and endothelial cells have been found to be elevated in sickle cell disease and PNH.

Microparticle Measurement

Different methodologies available for MPs measurement are as follows:

- Immunoassay by ELISA capture
- Flow cytometry.

Immunoassay by ELISA Capture

Sample Collection

- Blood collected in 0.109 M citrate anticoagulant (9 vol blood in 1 vol trisodium citrate), through a clean venipuncture, avoiding blood activation.
- Plasma is prepared by blood centrifugation at 1,500 g for 15 min at room temperature (18–25°C), then plasma decantation (PPP).
- Centrifugation of plasma (PPP) is done for 2 min at 13,000 g at room temperature (18–25°C).
- It is possible to keep the specimen frozen (various months/years).

Assay Principle

- Micro ELISA plate is coated with streptavidin and Annexin V then washed.
- Diluted tested plasma is introduced in a microwell, in presence of calcium.

- When present, microparticles (MPs) exposing anionic phospholipids bind to Annexin V.
- Following a washing step, first FXa and Va are introduced, with calcium, then prothrombin.
- Generated thrombin is measured using a specific chromogenic substrate, and absorbance is measured at 405 nm .

Results

- MPs in normal individuals are < 10 nmol/L.
- In various pathologies the concentration is 2 to 10 times those of normal plasma.

Advantages

- ELISA is available at most research institutions.
- It is less costly than flowcytometry.
- Not restricted by size as it measures all MPs including those that are < 0.4 or 0.1 μ).

Disadvantages

- Analysis is done in batches and thus takes longer time.
- No size determination is possible.

Flow Cytometry

- Flow cytometry relies on the antigenic composition of MPs and allows them to be enumerated according to their cellular origin.
- Only large microparticles (> 0.4 μ) are measured as the size and content are important parameters.
- The microparticle source, surface antigen expression and proportion in circulation are shown in Table 5.1.

Table 5.1: Microparticle source, surface antigen expression and proportion in circulation

Cellular source of MPs	*Marker*	*Proportion in circulation*
Platelets	CD61 (GPIIIa) CD63 CD62p (P-selectin) CD41	80–90%
Leucocytes	CD45	< 5%
Erythrocytes	Glycophorin A	5–10%
T helper cells	CD4	< 1%
T cytotoxic cells	CD8	< 1%
B cells	CD20	< 1%
Monocytes/macrophages	CD14	< 5%
Endothelial cells	CD62e (E-selectin)	< 5%

Advantages

1. Rapid turnaround time.
2. Both fresh and frozen specimens may be used.

3. The expression of two or more antigens on the MPs may be simultaneously demonstrated.
4. Easy method for quantification using commercial beads.

Disadvantages

1. The detection of particles less than 0.3 μm is difficult by flow cytometry.
2. Different machines have different sensitivities.
3. Centrifugation speeds for sample processing are variable and not standardized.

Other rarely used methods

- Functional assays
- Nanoparticle analysis
- Atomic force microscopy

MICROPARTICLES: GOOD OR BAD

- MPs are noxious elements, supporting proinflammatory, procoagulant potential and inhibiting vascular repair.
- Increased levels of MPs of endothelial origin in various pathologies, such as atherothrombosis, vasculitis, and sepsis have been seen.
- However, these EMPs have a beneficial effect on endothelial integrity, such as stimulation of vascular repair, control of cell death mechanisms and cytoprotective activities.
- Neutrophil-derived MPs release potent anti-inflammatory effectors (such as annexin A1) at the onset of an inflammatory response.

Recent Developments

- Some currently used therapies are known to affect microparticle generation.
- For example, abciximab, a glycoprotein IIb–IIIa receptor antagonist.
- It is currently used as an antiplatelet drug in prevention of ischemic complications after percutaneous coronary intervention.
- Also, it almost completely blocks platelet vesiculation *in vitro*.
- Treatment of patients suffering from transient ischemic attacks with calcium channel blockers has been shown to decrease microparticle generation.
- A randomized, placebo-controlled, double blind trial showed that treatment of patients with congestive heart failure with vitamin C decreased the number of circulating microparticles.
- Cilostazol (a selective cyclic AMP phosphodiesterase inhibitor and antiplatelet agent) and ticlopidine (an ADP antagonist) decrease platelet-derived MPs.
- Eposartan an antihypertensive agent, lowered or normalized numbers of monocyte and platelet derived MPs.

POINTS TO REMEMBER

- Cellular MPs are submicrometric fragments resulting from the remodeling of plasma membrane in response to numerous conditions, including activation and apoptosis.
- MPs may prove useful as circulating biomarkers of endothelial dysfunction and

prothrombotic state, both in disease and to monitor the effects of treatment, such as statins in vascular diseases.

- The understanding of pathophysiological relevance of blood MPs is still in its infancy.
- Future studies are needed to provide additional evidence of the role of MPs in various disease states.
- This may lead to development of additional interventions and strategies for the prevention and treatment of various disorders.

BIBLIOGRAPHY

1. Burnier L, Fontana P, Kwak BR, et al. Cell-derived microparticles in haemostasis and vascular medicine. Thromb Haemost. 2009;101:439–51.
2. Diamant M, Tushuizen ME, Sturk A, et al. Cellular microparticles: New players in the field of vascular disease? Eur J Clin Invest. 2004;34:392–401.
3. Dumaswala UJ, Greenwalt TJ. Human erythrocytes shed exocytic vesicles in vivo. Transfusion. 1984;24:490–92.
4. Horstman LL, Jy W, Bidot CJ, et al. Potential roles of cellderived microparticles in ischemic brain disease. Neurol Res. 2009;31:799–806.
5. Jy W, Ricci M, Shariatmadar S, et al. Microparticles in stored red blood cells as potential mediators of transfusion complications. Transfusion. 2011;51:886–93.
6. Kozuma Y, Sawahata Y, Takei Y, et al. Procoagulant properties of microparticles released from red blood cells in paroxysmal nocturnal haemoglobinuria. Br J Haematol. 2011;152:631–9.
7. Leroyer AS, Anfosso F, Lacroix R, et al. Endothelial-derived microparticles: Biological conveyors at the crossroad of inflammation, thrombosis and angiogenesis. Thromb Haemost. 2010;104:456–63.
8. McFaul SJ, Corley JB, Mester CW, et al. Packed blood cells stored in AS-5 become proinflammatory during storage. Transfusion. 2009;49:1451–60.
9. Rubin O, Crettaz D, Tissot JD, et al. Microparticles in stored red blood cells: Submicron clotting bombs? Blood Transfus. 2010;8(Suppl 3):s31–s38.
10. Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: Potentially pathogenic agents and diagnostic markers. Transfus Med Rev. 2006;20:1–26.

Recent Concepts in Tumor Immunology

6

Tanisha Singla

INTRODUCTION

Immune Surveillance

- Immune surveillance is a theory that the immune system patrols the body not only to recognize and destroy invading pathogens but also host cells that become cancerous.
- When a tumor escapes immune surveillance and grows too large for the immune system to kill, cancer occurs.

Evidence for Immune Reactivity to Tumors

- Tumors that have lymphocytic infiltration have a better prognosis than those that do not.
- Many lymph nodes draining tumor sites show reactive changes.
- Certain tumors regress spontaneously (e.g. melanoma, neuroblastoma).
- There is an increased incidence of primary and secondary malignancies in immunodeficient patients.
- The young and the very old have an increased occurrence of tumors.
- These members of the population often have an immune system that is less effective.

Tumor Antigenicity

- Next-generation sequencing has revealed that many somatic mutations occur in the DNA of cancer cells that were not known previously.
- These mutations occur in the coding regions of genes and are transcribed into RNA, which is then translated into proteins.
- Fragments of these mutated proteins **appear as peptides displayed by HLA molecules** on the surface of tumor cells, where they can be recognized as **"foreign" peptides** by T cells.
- These tumor antigens are useful tumor markers in identifying tumor cells and are potential candidates for use in cancer therapy.

IMMUNOGENICITY OF TUMORS

Two types of tumor antigens:

- **Tumor associated transplantation antigens (TAA):** These are found on tumor cells and on normal cells during fetal life (oncofetal antigens), after birth in selected organs at low concentration.
- **Tumor specific transplantation antigens (TSTA):** Present only on tumor cells, but not on normal cells
- Most chemically- or physically-induced tumors or those produced as a result of a virus, have **neoantigens**.
- Spontaneously occurring tumors are often weakly immunogenic or non-immunogenic.
- Antigenic changes observed in malignant cells include:
  - Reappearance of fetal antigens (oncofetal antigens)
  - Expression of unique antigens not expressed by normal cells.

Oncofetal Antigens

- Oncofetal antigens may appear due to de-repression of genes that were only expressed early in life.
- Two major oncofetal antigens are **alpha-fetoprotein** (**AFP)** and **carcinoembryonic antigen** (**CEA**).
- Others include—pancreatic oncofetal antigen, fetal sulfoglycoprotein.
- Studies have shown that expression of oncofetal antigens in adults is not limited to tumors.
- These proteins are increased in tissues and in the circulation in various **inflammatory conditions**, and they are also found in **normal tissues**.
- There is no evidence that oncofetal antigens are important inducers or targets of antitumor immunity.
- However, they can serve as markers that aid in tumor diagnosis and clinical management

Tumor Antigens Produced by Oncogenic Viruses

- Viruses produce proteins that are recognized as foreign by the immune system.
- The most potent of these antigens are proteins produced by latent DNA viruses.
- Example: Human papillomavirus (HPV) E6 and E7 proteins in cervical cancer.

Products of Mutated Genes

- Neoplastic transformation occurs due to genetic alterations in proto-oncogenes and tumor suppressor genes.
- These mutated genes encode variant proteins which are recognized by the immune system as nonself.
- Example: Mutated oncoproteins like RAS and BCR/ABL fusion proteins.
- Mutated tumor suppressor gene products like mutated p53 protein.

Altered Cell Surface Glycolipids and Glycoproteins

- Tumors express higher than normal levels and/or abnormal forms of surface glycoproteins and glycolipids, which may be diagnostic markers and targets for therapy, e.g. CA-125 and CA-19–9 expressed on ovarian carcinomas.

Cell Type-specific Differentiation Antigens

- Tumors express molecules that are normally present on the cells of origin. These antigens are called ***differentiation antigens*** because they are specific for particular lineages.
- These are normal self-antigens therefore they do not induce immune responses.
- They are potential targets for immunotherapy and for identifying the tissue of origin of tumors.
- Antibodies against CD20 (Rituximab) can be used in the treatment of B cell leukemia and lymphoma.

IMMUNITY AGAINST TUMORS

- Evidence for immunity against malignancy comes mostly from experimental models.
- In experimental studies, animals can be immunized by administering inactivated tumor cells or by removal of a primary tumor.
- Immunity can be transferred from an animal, in which a tumor has regressed, to a naive animal by injection of lymphocytes (T cells).

MECHANISMS OF IMMUNE SURVEILLANCE

- All components take part- NK cells, cytotoxic T lymphocytes (CTL) macrophages.
- Out of these macrophages play predominant role.

Natural Killer Cells (NK) and Antibody-Dependent Cellular Cytotoxicity

- NK cells are effector cells in an innate immune response.
- They play a dynamic role in the nonspecific killing of tumor cells or virus-infected cells
- NK cells can be moved to kill target cells that do not express MHC class I molecules.
- Two cell surface molecules, CD16 and CD56, are commonly used to identify NK cells.
- CD16 is an Fc receptor for IgG, and it confers on NK cells the ability to lyse IgG-coated target cells.
- This phenomenon is known as **antibody-dependent cell-mediated cytotoxicity (ADCC).** (The function of CD56 is not known)
- The functional activity of NK cells is regulated by a balance between signals from activating and inhibitory receptors.
- There are many types of activating receptors, of which the **NKG2D family** is the best characterized.
- The NKG2D receptors recognize surface molecules that are induced by various kinds of stress, such as infection and DNA damage.
- NK cell inhibitory receptors recognize self Class I MHC molecules, which are expressed on all healthy cells. The inhibitory receptors prevent NK cells from killing normal cells.
- Virus infection or neoplastic transformation enhances expression of ligands for activating receptors and reduces the expression of class I MHC molecules.
- As a result, the balance is tilted toward activation, and the infected or tumor cell is killed.

T Lymphocytes and Antitumor Immunity

- Effector mechanisms of tumor cell killing by cytotoxic T cells (CTLs) are as follows:
  - They can directly facilitate the lysis of tumor cells by identifying distinctive antigens presented by tumor cells.
  - They can kill tumor cells by signaling the induction of apoptosis in the target cells and by the secretion of perforins and granzymes.

B Lymphocytes and Antibodies

- The production of antitumor cell antibodies does not play a dominant role in host antitumor immune responses.
- However, monoclonal antibodies that are responsive to tumor-associated antigens show usefulness in antitumor therapy and for tumor detection.

Macrophages, Monocytes, and Dendritic Cells

- Monocyte/macrophages play important roles in immune responses.
- Macrophages, which form an important part of innate immune responses, also play a key role in the generation of adaptive immune responses, because they can act as active ***antigen-presenting cells***.
- Helper (CD4) T and CD8 T lymphocytes can be activated by antigen-presenting macrophages that show processed antigen joint with self MHC molecules.
- Antigen presenting cells also deliver key costimulatory signals that are important for T-lymphocyte activation.
- Macrophages can also ingest and kill microorganisms, and can act as cytotoxic, antitumor killer cells.
- Macrophages and monocytes produce various **cytokines and chemokines**, which are involved in both innate and adaptive immune reactions.
- These chemokines and cytokines have straight biologic effects on tumor cells - both as growth-inducing and growth-inhibiting factors.
- **Mature dendritic cells** are very effective antigen-presenting cells for T cells.
- They express very high levels of MHC class I and class II molecules, along with high levels of cell surface lymphocyte stimulatory molecules and adhesion molecules.
- They also secrete chemotactic factors (chemokines) that recruit T cells.

FAILURE OF IMMUNE SURVEILLANCE

- Cancers represent a rare failure of a system that has been eliminating transformed cells throughout life.
- If a cell succeeds in starting down the path leading to uncontrolled mitosis, it may acquire somatic mutations that protect it from attack by the host's immune system **(cancer immunoediting).**
- Examples:
  - Reduced expression of tumor antigens.
  - Reduced expression of class II MHC molecules needed to display tumor antigens to $CD4^+$ Th cells.
  - Reduced expression of class I MHC molecules needed to display tumor antigens to $CD8^+$ cytotoxic T lymphocytes (CTLs).
  - Reduced efficiency of loading antigenic peptides into MHC molecules.

- Secretion of immunosuppressive cytokines (TGF-β, IL 10).
- Recruitment of immunosuppressive T regulatory cells.

Can the immune system be manipulated to target cancer?

- The rapidly advancing field of cancer immunology has produced several new methods of treating cancer, called immunotherapies, that increase the strength of immune responses against tumors.
- Immunotherapies either stimulate the activities of specific components of the immune system or counteract signals produced by cancer cells that suppress immune responses.

IMMUNOTHERAPY

Immune Checkpoint Modulators

- Immune checkpoint modulators are proteins that normally keep immune responses in check by preventing overly intense responses that might damage normal cells as well as abnormal cells.
- Some tumors can commandeer these proteins and use them to suppress immune responses.
- Blocking the activity of immune checkpoint proteins releases the **"brakes"** on the immune system, increasing its ability to destroy cancer cells.
- The first drug to receive FDA approval, I**pilimumab (Yervoy®)**, for the treatment of melanoma, blocks the activity of a checkpoint protein known as CTLA4, which is expressed on the surface of cytotoxic T lymphocytes.
- CTLA4 acts as a "switch" to inactivate these T cells, thereby reducing the strength of immune responses.
- **Mechanism:** Cancer cells produce antigens which are recognized by dendritic cells and are presented to cytotoxic T cells which are then destroyed.
- However along with the antigens, the dendritic cells also present inhibitory signals that binds to CTLA-4 receptor present on cytotoxic T cell which turns **off** the cytotoxic mechanism leading to survival of the tumor cells.
- Ipilimumab binds to CTLA-4 receptor, thus blocking the inhibitory signal.
- Two other FDA-approved checkpoint inhibitors-nivolumab (Opdivo® and pembrolizumab (Keytruda®), work in a similar way, but they target a different checkpoint protein-**PD-1 (programmed cell death protein 1)**.
- Nivolumab is approved to treat patients with malignant melanoma and lung cancer.
- Pembrolizumab is approved to treat patients with malignant melanoma.

Adoptive Cell Transfer: CARs and TILs

- Another form of immunotherapy that is being actively studied is called adoptive cell transfer (ACT).
- In several small clinical trials testing ACT, some patients with very advanced cancers primarily **melanoma and blood cancers like leukemia and lymphoma** have had their disease completely eradicated.
- In ACT the patient's autologous T cells are expanded and manipulated *ex vivo* which are then reinfused into the patient to exert anti-tumor response.

CAR T cell Therapy

- In this form of ACT, patients' T cells are collected from the blood.
- These T cells are then genetically modified in the laboratory to express a synthetic receptor on their surface known as a **chimeric antigen receptor (CAR)**.
- CARs are of two types—**extracellular** single-chain variable fragments (ScFv) for antigen recognition and **intracellular** signaling domains for activating T cells.
- In CAR-T cells, the extracellular domain ScFv is responsible for assigning the specificity of CTLs to the malignant cells.
- CAR intracellular signaling domains provide the necessary signals for T cell activation.
- After the immune cells are engineered to express a CAR, they are then grown in the laboratory until there are hundreds of millions of them.
- The entire batch of CAR T cells is infused into the patient in a single dose.
- Due to the specificity to bind to the cancer cells - the modified T cells attack these cells.
- In 2017, **FDA approved the first CAR T cell therapy, tisagenlecleucel (Kymriah™)** for children and adolescents with acute lymphoblastic leukemia.

Tumor Infiltrating Lymphocyte (TIL) Therapy

- In this form of ACT-immune cells are collected from a patient's tumor-called tumor-infiltrating lymphocytes (TILs)
- TILs are immune cells that have naturally entered a tumor and their presence is thought to indicate that the immune system is trying to attack the cancer.
- Unlike CARs they do not undergo any further modifications or engineering.
- As with CARs and TCRs, large populations of these TILs are grown in the laboratory.
- The expanded TILs are then activated by treatment with cytokines.
- Then the activated cells are infused into the patient in a single dose.
- The idea behind this approach is that the TILs have already shown the ability to target tumor cells, but there may not have been enough of these immune cells around the tumor to eradicate it.
- Introducing massive amounts of activated TILs can help to overcome these barriers, leading to tumor destruction.

THERAPEUTIC ANTIBODIES

- Therapeutic antibodies are antibodies made in the laboratory that are designed to cause the destruction of cancer cells.

Monoclonal Antibodies in Immunotherapy (Magic Bullet)

- Monoclonal anti-tumor antibodies are used in different forms for the treatment of cancer.
- They are also used as vehicles to target anticancer drugs and toxins.
- Specific antibodies are also used in the diagnosis of metastatic lesions, which are not detectable by conventional radiological means.

IMMUNOCONJUGATES

- Tumor-targeted immunoconjugates consist of an **antibody and an effector moiety** which are bonded together.

- If the effector moiety is a cytotoxic drug, it is called **antibody–drug conjugate (ADC).**
- If the effector moiety is a protein toxin, it is called an **immunotoxin.**
- If the effector moiety is a radionuclide (toxic radioactive isotope), it is called a **radioimmunoconjugate.**
- Antibody drug conjugates consist of a cytotoxic drug conjugated to an antibody for targeting a specific antigen.
- The antibody binds to the specific antigen and is internalized by receptor-mediated endocytosis.
- Free drug is released in the intracellular compartment by lysozomal enzyme-mediated degradation of the drug–antibody conjugate whereby the drug restores its cell-killing potential
- USFDA has approved several ADCs for the treatment of patients with cancer, including:
  - Ado-trastuzumab emtansine **(**Kadcyla®) **for the treatment of breast cancer**
  - Brentuximab vedotin (Adcetris®) **for Hodgkin lymphoma**
  - Ibritumomab tiuxetan (Zevalin®) **for non-Hodgkin B cell lymphoma.**

Complement Binding Antibodies

- Monoclonal antibodies—Herceptin or Rituximab, cause selective cellular toxicity by binding to specific target antigen followed by cell lysis either by **antibody-dependent cellular cytotoxicity** or by complement activation and **complement-dependent cytotoxicity**.
- Rituximab (Rituxan®) targets CD20 which is expressed on surface of B cells.
- Used in the treatment of B cell lymphoma and leukemia.

Immunotoxins

- Immunotoxins are composed of a protein toxin attached to an antibody.
- Toxins are mainly cytotoxic enzymes. The antibody recognizes and binds to the specific tumor antigen and gets internalized.
- After the conjugate is internalized, the attached toxin begins its cytotoxic function and causes cell death.
- Example: Denileukin diftitox (**ONTAK®**) is approved for the treatment of cutaneous T cell lymphoma, contains a toxin produced by the bacterium *Corynebacterium diphtheriae*.

Antibody-Directed Enzyme Prodrug Therapy

- This includes the activation of a weakly toxic prodrug by an enzyme attached to the antibody at the tumor site to an active toxic drug.
- Results in humans have not been as positive as in experimental animals, but efforts continue to develop this approach into an effective therapeutic approach.

Cancer Stem Cells

- The existence of a small population of cancer cells called cancer stem cells which differentiate to form cancer cells may require targeting through **stem cell specific antigen** for specific eradication of these subtypes of cancer cells.

- Relapse of tumor cells after chemotherapy is associated with a small population of stem cells left behind unaffected by chemotherapy.
- These cells differentiate later to become the cancer cells which are resistant to previous therapy.
- Since stem cells are not rapidly dividing cells, conventional chemotherapy targeting rapidly dividing cells remains ineffective against cancer stem cells.
- Hence for a complete eradication of cancer, it is important to eradicate cancer cells along with cancer stem cells.
- This requires the identification of specific targets or exclusive pathways non-existent in normal cells but prevalent in cancer stem cells.
- This is still a developing concept and more research is going on.

Nonspecific Biological Products

- A variety of immuno-potentiating agents (**biological response modifiers**) are used to enhance anti-tumor immunity.
- They include:
  - bacterial products
  - synthetic chemicals
  - cytokines.
- Most of these agents exert their effects by activating macrophages and natural killer (NK) cells, eliciting cytokines or enhancing T cell functions.

POINTS TO REMEMBER

- Many mechanisms of the immune system can have active antitumor responses, including direct cytotoxicity directed against tumor cells along with the production of immune-enhancing cytokines.
- The mis-regulated expression of various genes, including overexpression of oncogenes, loss of anti-oncogenes and overproduction of growth factor and cytokines may be responsible in the development and progression of some cancers.
- Alteration of the environment and antitumor immune responses may also play a role in the development and growth of malignancies.
- Understanding the exact strategies that are involved in the development and growth of cancer will provide chances for the lucid design of effective antitumor immunotherapy.
- Several forms of experimental immunotherapy for cancers have been studied, including biologic response modifiers and cytokines.
- In recent years, great advances have been made in the development of suitable forms of monoclonal antibodies directed to cell-surface molecules expressed on tumor cells.
- Immunologic therapy of several types of cancer currently remains experimental.
- These therapies can expand what can be achieved with ideal chemotherapy and therefore could deliver an incremental development in patient management over current standards.

BIBLIOGRAPHY

1. AJ Simpson, OL Caballero, A Jungbluth, YT Chen, LJ OldCancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer. 2005; 5: pp. 615–25.
2. B Gaugler, N Brouwenstijn, V Vantomme, JP Szikora, CW Van der Spek, JJ Patard, et al. A new gene coding for an antigen recognized by autologous cytolytic T lymphocytes on a human renal carcinoma. Immunogenetics. 1996;44: pp. 323–30.
3. C Traversari, P van der Bruggen, IF Luescher, C Lurguin, P Chomez, A Van Pel, et al. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E J Exp Med. 1992;176: pp. 1453–57.
4. P Boel, C Wildmann, ML Sensi, R Brasseur, JC Renauld, P Coulie, et al. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes Immunity. 1995;2: pp. 167–75.
5. P van der Bruggen, C Traversari, P Chomez, C Lurquin, E De Plaen, B Van den Eynde, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma Science. 1991;254: pp. 1643–47.
6. U Sahin, O Tureci, H Schmitt, B Cochlovius, T Johannes, R Schmits, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA. 1995;92: pp. 11810–13.

7 Connexins and their Role in Tumour Biology

Harsh Batra

INTRODUCTION

- Cells in a tissue are connected to each other through intercellular junctions which can be of five types:
  i. Tight junctions
  ii. Desmosomes
  iii. Hemidesmosomes
  iv. Adherens junctions
  v. Gap junctions
- Existing in nearly every mammalian cell, ***Connexins***, or gap junction proteins are family of structurally related transmembrane proteins that assemble to form vertebrate gap junctions.
- Gap junctions form a few to hundred tightly packed channels that allow direct exchange of small molecules between adjoining cells.
- The diversity of function is attributed to the subset of connexins that are expressed in any one cell type.

NOMENCLATURE

- Connexins are abbreviated as either Cx or CX.
- They are named according to their molecular weights, e.g. Cx26 is the connexin protein of 26 kDa.
- However, this may lead to confusion when connexins from different species are compared, e.g. human Cx36 is homologous to zebrafish Cx35.
- Another system of nomenclature based on sequence similarity and length of the cytoplasmic domain.
- It designates connexins as GJ (for gap junction) α, β, and γ (subgroups).
- They are serially numbered in the order of their discovery within each subgroup. For example: CX43, was the first connexin discovered in the α subgroup.
- It is, therefore, designated as Gjα1.
- Cx32 was the first in the β subgroup: Gjβ1.
- A Gap Junction Conference (2007) was held in Elsinore, Denmark.
- It was decided to use the GJ nomenclature system for the genes that encode connexins.

- The participants wished to retain the connexin nomenclature for the encoded proteins using the weight of the human protein.

GENERAL PRINCIPLES ASSOCIATED WITH CONNEXINS

- Many tissues and cell types express two or more members of the connexin family.
- For example, keratinocytes express at least Cx26 (connexin26), Cx30, Cx30.3, Cx31, Cx31.1 and Cx43.
- Co-expression of multiple connexin family members within the same cell type allows for possible compensatory mechanisms to overcome the loss or mutation of one connexin family member.
- This principle has been demonstrated effectively in connexin-gene-ablation studies.
- It was observed that the prevalence of disease or the incidence of abnormal development is far less than might be predicted if no compensatory mechanisms existed between co-expressed connexin family members.
- In a study by Kelsell et al. it was shown that humans suffering from loss-of-function mutations in Cx26 are deaf.
- But no accompanying liver disease was reported since Cx26 and Cx32 are co-expressed in hepatocytes.
- Though two or more connexins may be co-expressed in the same cell, the resulting channels formed cannot always compensate for the loss or mutation of a connexin family member.
- For example, in skin, a subset of loss-of-function Cx26 mutations that result in deafness also manifest as skin disease.
- This is despite the fact that the epidermis is rich, with presence of multiple members of the connexin family.
- ***The most ubiquitously expressed connexin is Cx43.***
- Cx43 is endogenously expressed in at least 35 distinct tissues encompassing over 35 cell types.
- These include cardiac myocytes, keratinocytes, astrocytes, endothelial cells and smooth-muscle cells.

MOLECULAR STRUCTURE OF CONNEXINS

- Connexins are polytopic integral membrane proteins where the polypeptide backbone threads through the membrane four times (transmembrane).
- This yields:
  1. Two extracellular loops (EL-1 and EL-2).
  2. A cytoplasmic loop (CL) with both the N-terminus (AT) and the C-terminus (CT) exposed to the cytoplasm.
- The protein is made up of nine domains as follows:
  - N-terminus
  - Two extracellular loops (EL)—stabilized by intramolecular disulfide bridges
  - Four transmembrane domains
  - Cytoplasmic loop domain
  - C-terminus domain.
- Amongst these nine domains, sequence conservation is most evident within the N-terminus, two EL domains and four transmembrane domains.

- Conversely, the cytoplasmic loop and the C-terminus show high diversity in terms of size and post-translational modification.
- Connexins oligomerize into characteristic hexamers also known as '***connexons***' or 'hemichannels'.
- This helps in gap junction channel formation where two connexons of adjacent plasma membranes appose and dock against each other.
- Individual gap-junction channels are arranged in hexagonal arrays also called ***gap-junction plaques.***
- Diameter of channel is approximately 2 nm.
- Possible channel subtypes increase exponentially when two compatible connexins are co-expressed in the same cell and are capable of assembling both in homomeric and heteromeric fashion.
- For example, heteromeric Cx26/Cx32 connexons exist in the liver; Cx46/Cx50 connexons in the lens; Cx26/Cx30 connexions in the cochlea.
- Thus there can be homotypic, heterotypic and combined heterotypic/heteromeric arrangements.
- These permutation and combinations of connexins help in the diversity of functions in a particular cell type.
- Channels formed of heteromeric connexons have different properties from those of homomeric channels.
- Properties of heteromeric channels are manipulated by regulating the ratio of constituent connexins.
- This allows regulation of the permeability and conductance of gap junctions.

Biosynthesis

- Having a short half-life of only a few hours, connexins are regularly biosynthesized and degraded.
- They have a short half-life in order to respond to physiological requirements to either up- or down-regulate the extent of gap-junction coupling.
- This response mechanism is best exemplified in the myometrium, where it is proposed that steroid hormones promote the dramatic 5-fold increase in total gap junctions just prior to labor onset.
- Reciprocally, they are rapidly cleared following labor re-establishing a steady-state level in the uterus.
- Connexins are synthesized on ER-bound ribosomes and inserted into the ER cotranslationally followed by folding of the connexin protein while in the ER.
- Connexin oligomerization occurs throughout the transport of the connexons within the ER till the trans-Golgi network, where oligomerization is completed.
- Connexon hemichannels are packaged into vesicles (between the ER and the trans-Golgi network) and delivered to the membrane which can be microtubule dependent or independent.
- Most get inserted and interact via their extracellular loops to form intercellular channels.
- The individual channels aggregate in the membrane to form plaques or gap junctions.
- Some connexon hemichannels may remain uncoupled playing a role in cell life cycle.

Post-translational Modification

- Connexins may undergo various types of post-translational modifications, including phosphorylation, hydroxylation, acetylation, disulfide binding, nitrosylation, and palmitoylation.
- The most studied is phosphorylation.
- It is essential for proper control of formation and modulation of function of these channels.
- Phosphorylation by different kinases such as Src, PKC, and MAPKs is required for and affects connexin/connexon trafficking, assembly and disassembly, degradation, and gating (rapid opening and closing) of gap-junction channels.
- Effect of phosphorylation on channel gating is very specific.
- For example, phosphorylation of a connexin isoform such as Cx43 on different residues by the same kinase may lead to opposite effects with respect to enhancing or inhibiting gap junction function.
- Other connexin functions including control of growth and proliferation are also affected.

Half-life and Degradation

- Connexin proteins have a very short half-life of a few hours.
- Synthesis and delivery to the membrane is coupled to simultaneous gap-junction internalization and degradation.
- Newly delivered connexions localize to the periphery of the existing gap junctional plaques and "old" connexons to be degraded are present at the center of the plaque.
- Following internalization, these complexes undergo preliminary degradation and finally disassembly of gap junctions and connexions into individual connexions.
- Connexin proteins undergo complete degradation through proteasomes or lysosomes.

Connexin Interactions

- These proteins do not act in isolation in gap-junction complexes.
- They interact with many associated proteins that play essential roles in regulating the assembly, function, and life-span of connexins.
- The main interacting partners include cytoskeletal elements, junctional proteins and enzymes.

Interaction with Cytoskeleton

- Cytoskeleton elements including *Microtubules*, *Actin*, *Spectrin* and *Debrin* help in transporting connexins to the membrane and for their rapid turnover and replenishment.
- Cx43 directly binds to α- and β-tubulin.
- Microtubules at the cell-periphery co-localize with Cx43-based gap junctions.
- Interaction with microtubules is essential in allowing directed transport of newly synthesized connexion hemichannels to the plasma membrane.
- Microtubules also direct newly synthesized connexions to sites of pre-existing adherens junctions.
- Interactions of connexins with the actin cytoskeleton helps to stabilize gap junctions at the plasma membrane.

- Debrin (developmentally regulated brain protein) is a novel connexin-interacting cytoskeletal protein.
- It is an actin binding protein that mediates cellular polarity and formation of stabilized plasma membrane domains.
- It interacts with the carboxy-terminal end of Cx43 and stabilizes Cx43 gap junctions at the membrane.

Interaction with Junctional Proteins

- Interaction of gap junction proteins with proteins of the adherens and tight junctions leads to formation of large protein intercellular complexes containing multiple junctional molecules.
- These interactions form a regulatory backbone for various stages of the connexin life cycle—membrane insertion, localization, and plaque formation.
- For example, Zonula Occludens-1 (ZO-1) is a tight junction membrane-associated guanylate kinase which is involved in the organization and trafficking of gap junctions.
- ZO-1 has also been shown to regulate Cx43 mediated gap junctional communication in osteoblastic cells by altering its membrane localization.
- The C-terminal of Cx43 and Cx45 interact with the second PDZ domain of ZO-1, thereby playing a role in recruitment of regulatory protein to gap junctions.
- Furthermore, connexions have also been shown to have important interactions with occludin, claudin and cadherin.

Interaction with Enzymes

- Tyrosinases, serine/threonine kinases and phosphatase enzymes have been shown to have important interaction with connexins.

FUNCTIONS OF CONNEXINS

- They can be broadly classified as:
  - *Gap junction-dependent functions*
  - *Hemichannel-dependent functions*
  - *Gap junction and hemichannel-independent functions*

Gap Junction-Dependent Functions

- Gap junction intercellular communication (GJIC) is a central conduit of ions, essential metabolites, and second messengers like Ca^{2+}, cAMP, cGMP and IP between adjacent cells.
- It plays an active role in intercellular signaling and communication.
- Gap junction channels, alternate between "closed" and "open" conformations.
- This is regulated by the following mechanisms:
  - Calcium concentration
  - pH
  - Trans-junctional potential
  - Protein phosphorylation.
- The regulatory sites that are responsive to pH levels are found in the intracellular loop and C terminus domains of the connexin proteins.

- These regions show a little sequence homology between different connexins.
- Hence, differential composition of the gap junction channels determine response to pH in different cell types.
- **Transmembrane voltages:** Large transjunctional voltages close the channels, possibly through the C-terminus interacting with the pore of the channel.
- Phosphorylation status changes the number of gap junctions at the cell–cell interface.
- This happens either through controlling connexin trafficking and degradation or by completely closing or opening the channel for passage of molecules.
- Gap junction are involved in developmental and regulatory events such as embryonic growth, bone modeling, alveolar differentiation, CNS signaling and neural functions.
- Proper gap junction intercellular communication is essential for differentiation of various tissues, including the mammary gland, lens, bone marrow, Sertoli cells, adipocytes, and other cells and organs.
- Restoration of gap junctional communication in colon cancer cells leads to re-establishment of a differentiation phenotype.
- The tumor cells show increased levels of connexin 43 protein and its phosphorylation status.
- Gap junctions also have a role in cell death.
- **Bystander effect,** i.e. gap junctions spread a death signal between dying cells and those adjoining them, which is mediated by calcium influx between these cells.
- For example, spread of injury in the brain following hypoxia/ischemia.
- GJ have also been shown to "rescue" dying cells.
- It is achieved by ensuring passage of substrates like ATP, glucose, ascorbic acid, etc. from one cell to other.
- They also inhibit the passage of cytotoxic agents such as nitric oxide and others.

Hemichannel-dependent Functions

- Involved in various aspects of cell life, including calcium signaling, cell proliferation and death, as well as the normal functioning and development of various cell types.
- Osmoregulatory role in ventricular myocytes.

Gap Junction and Hemichannel-independent Functions

- Connexins also play various roles independent of their channel-forming properties.
- These are mediated through their multiple interacting partners.
- Subsequently leading to the modulation of gene expression resulting in a wide range of effects.
- This is particularly important with respect to their role in tumor biology.

ROLE IN TUMOR BIOLOGY

- Connexins are implicated in the carcinogenic process.
- Disruption of normal connexin functions is a hallmark of many tumors.
- Alteration of connexin expression (over-expression/deletion) leads to changes in gene expression in multiple pathways and cellular functions.
- Cx26, involved in tumor suppression, inhibits cell migration and invasion in a gap-junction independent mechanism in the MDA-MB435 tumor cell line through regulation of β1-integrin and MMP levels.

- It also reverses the malignant phenotype of MCF-7 breast cancer cells.
- Breast tumor cell lines exhibit down-regulation of connexin gene expression.
- Deficiency of Cx43 gap junction is an independent adverse marker for breast cancer.
- Glioblastoma (GBM) with high mitotic index have been shown to have a reduced expression of Cx43.
- Cx32 suppresses growth, invasion, and metastasis of RCC cell lines.
- It is done through various modulators like tight junction proteins, VEGF, etc.
- Controlled expression of Cx43 prevented cell growth independent of its channel forming properties.
- This is through the association of its C-terminal domain with proteins such as ZO-1.
- Alteration of connexin expression that is over-expression/suppression leads to changes in gene expression in multiple pathways and cellular functions as follows:
  - Transcription
  - Metabolism
  - Cell/cell and cell/ECM adhesion
  - Cellular signaling
  - Transport
  - Cell cycle and division.
- One such mechanism is through connexin-responsive elements (CxRE).
- Gap junctional communication induces differential recruitment of sp1 and sp3 transcription factors to the CxRE through the PI3K pathway.
- This regulates expression of genes having this promoter element.
- This effect of connexins on gene expression has also been observed in many studies showing re-expressing connexons in connexin-deficient tumors.
- This affects growth and tumorigenicity of the tumor cells via gap junction-dependent and independent mechanisms.
- It includes regulation of gene expression of proteins involved in the various processes, such as MMPs and TIMPs, SKP2 and p21.
- However, in metastasis, heterocellular gap junctions between tumor cells and cells of the secondary tumor site or lymph nodes increase possibility of metastasis.
- For example, expression of Cx26 and Cx43 is increased in breast cancers with lymph node metastasis as compared to primary tumor confined to the breast.
- Cx43-mediated GJIC results in increased diapedesis of the breast tumor cell line HBL100.
- Heterocellular gap junctions between tumor cells and cells of the secondary tumor site or lymph nodes lead to increased possibility of metastasis.
- Other studies have observed that re-expression of homocellular connexions in metastatic tumor cells results in decreased tumor metastatic potential.
- Suppression of MDA-MB-435 tumor cell line metastatic potential results in increased homocellular GJIC.
- There is also a change in the expressed connexion profile.
- Untreated cells expressed only Cx32.
- Metastasis-suppressed cells expressed Cx43.
- Breast cancer and melanoma cells utilize connexions (Cx43, Cx26) to initiate brain metastatic lesion.
- Activation of twist metastatic gene in breast cancer cells increases Cx43 expression which helps in extravasation, blood vessel co-option and colonization of cancer cells.

- This phenomenon is reversed in hepatocellular carcinoma cells where Cx43 enhances tumor cell malignancy through inhibition of Cx32-mediated GJIC and suppression of Cx32 expression altogether.
- Therefore, the impact of GJIC on tumor metastasis may be dependent on the type of connexion expressed and the type of cells involved.
- The roles of various connexins in cancer is summarized in Table 7.1.

Table 7.1: Important connexins and their role in cancer

Connexin	*Cancer*	*Function*	*Tumor activity*
Connexin 25	Leukemia	GJIC	Pro-tumorigenic
Connexin 26	Breast, cervical	GJIC	Anti-tumorigenic
	Cervical	Hemichannel activity	Anti-tumorigenic
	Breast	Protein-connexin interaction	Pro-tumorigenic
Connexin 30	Glioma, gastric	GJIC	Anti-tumorigenic
Connexin 31.1	Head and neck squamous cell carcinoma	GJIC	Anti-tumorigenic
	Non-small cell lung cancer	Protein-connexin interaction	Anti-tumorigenic
Connexin 32	Breast	GJIC	Pro-tumorigenic
	Renal cell carcinoma, ovarian	Protein-connexin interaction	Anti-tumorigenic
Connexin 36	Cervical	Protein-connexin interaction	Pro-tumorigenic
Connexin 37	Liver, insulinoma	Protein-connexin interaction	Pro-tumorigenic anti-tumorigenic
Connexin 43	Brain	GJIC	Pro-tumorigenic
	Breast	Hemichannel activity	Anti-tumorigenic
	Ovarian	Protein-connexin interaction	Anti-tumorigenic
Connexin 46	Brain	GJIC	Pro-tumorigenic
Connexin 50	Cervical	GJIC	Pro-tumorigenic

POINTS TO REMEMBER

- Gap junctional intercellular communication (GJIC) is a form of cell–cell communication mediating the exchange of small molecules between neighboring cells.
- Gap junctions (GJs) are formed by connexins (Cxs), and are subject to tight and dynamic regulation.
- They are involved in the cell cycle, differentiation, and cell signaling.
- The loss of Cxs and GJs is a hallmark of carcinogenesis, while their induction in cancer cells leads to a reversal of the cancer phenotype, induction of differentiation, and regulation of cell growth.
- An overexpression in heterocellular gap junction has been shown to have a role in tumor metastasis.
- They may also serve as novel targets for anti-tumor therapy.

BIBLIOGRAPHY

1. Belousov AB, Fontes JD, Freitas-Andrade M, Naus CC. Gap junctions and hemichannels: communicating cell death in neurodevelopment and disease. BMC Cell Biol. 2017;18:4.
2. Brownlee C. Role of the extracellular matrix in cell–cell signalling: paracrine paradigms. Curr Opin Plant Biol. 2002;5:396–401.
3. Dbouk HA, Mroue RM, El-Sabban ME, Talhouk RS. Connexins: a myriad of functions extending beyond assembly of gap junction channels. Cell Commun Signal. 2009;7:4.
4. Dorshkind K, Green L, Godwin A, Fletcher W. Connexin-43-type gap junctions mediate communication between bone marrow stromal cells. Blood. 1993;82:38–45.
5. Esseltine JL, Laird DW. Next-generation connexin and pannexin cell biology. Trends Cell Biol. 2016;26:944–55.
6. Giancotti FG, Ruoslahti E. Integrin Signaling. Science. 1999;285:1028–33.
7. Miyoshi K, Shillingford JM, Smith GH, Grimm SL, Wagner K-U, Oka T, et al. Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. J Cell Biol. 2001;155:531–42.
8. Oyamada M, Oyamada Y, Takamatsu T. Regulation of connexin expression. Biochim Biophys Acta Biomembr. 2005;1719:6–23.
9. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia. 2006;20:1487–95.
10. Vinken M, Vanhaecke T, Papeleu P, Snykers S, Henkens T, Rogiers V. Connexins and their channels in cell growth and cell death. Cell Signal. 2006;18:592–600.

Lymphoma Microenvironment and Immunotherapy

8

Ravi Pratap Singh

INTRODUCTION

- Lymphoma microenvironment is a dynamic and interactive supporting network of immune cells, stromal cells, cytokines, blood vessels and extracellular matrix.
- Composition of lymphoma microenvironment is guided by neoplastic cells. The microenvironment influences tumor initiation, progression and drug resistance.

COMPONENTS OF LYMPHOMA MICROENVIRONMENT

- **Immune cells**
  - Cytotoxic T cells (CTLs)
  - Follicular B helper T cells (TFH)
  - Regulatory T cells (Tregs)
  - Natural killer cells (NK)
  - Bystander B cells
- **Stromal cells**
  - Mesenchymal stromal cells (MSCs)
  - Lymphoma associated macrophages (LAMs)
  - Myeloid-derived suppressor cells (MDSCs)
  - Dendritic cells
- **Extracellular components**
  - Extracellular matrix (ECM)
  - Cytokines/chemokines
  - Lymphoma exosome.

ANGIOGENESIS

Mesenchymal Stromal Cells (MSCs)

- MSCs have both anti-inflammatory as well as immunosuppressive properties.
- MSCs differentiate into the fibroblastic reticular cells and follicular dendritic cells necessary for the infiltration of follicular lymphoma in the bone marrow.

MSCs in Lymphomas

- Marrow MSCs from patients with follicular lymphoma overexpress chemokine (C-C motif) ligand 2 (CCL2) resulting in growth sustenance of malignant B cells.

Research and Studies

- Coinjection of MSCs with neoplastic B cells promotes B cell lymphoma growth in the lacrimal glands of immunocompetent mice and are found to be associated with marked increase in CD4+ forkhead box P3 (FoxP3) + T cells and myeloid-derived suppressor cells.

LYMPHOMA-ASSOCIATED MACROPHAGES (LAMs)

LAMs in Hodgkin Lymphoma

- In advanced stage classic Hodgkin lymphoma (CHL) as well as in meta-analyses, a high-density of LAMs is a strong predictor of adverse outcomes in adult patients.
- In CHL, an **increased number of CD68 +** LAMs result in:
  - *Shorter progression-free survival*
  - *Increased likelihood of relapse after stem cell transplantation*
  - *Overall shortened disease-specific survival.*

Therapeutic Developments

- Immunomodulatory drugs such as pomalidomide convert the polarization status of macrophages from M2 to M1 in mouse models of central nervous system (CNS) lymphoma.
- This reduces signal transducer and activator of transcription (STAT) 6 signaling while enhancing STAT1 signaling resulting in increased phagocytic activity of macrophages.
- LAMs are a potential stratification biomarker.

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)

- Tumors recruit MDSCs by secreting factors like granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), and interferon gamma (IFN-γ).
- MDSCs suppress immune surveillance, particularly in the bone marrow.
- MDSCs have been shown to form mature osteoclasts in response to nuclear factor KB ligand (RANKL), increasing bone resorption.
- All these changes thereby influence the ability of tumors to spread into the marrow niche.

MDSCs in Hodgkin Lymphomas

- In CHL at initial diagnosis, all subsets of MDSCs were higher compared to controls. While the patients undergoing therapy showed decreased MDSC subsets.
- Patients with complete response had lower CD34+ MDSCs, monocytic MDSC and polymorphonuclear MDSCs
- Non-responders are seen to have higher levels.

MDSCs in Non-Hodgkin Lymphomas

- CD14 positive monocytes in patients with NHL showed reduced HLA-DR expression.
- This is associated with decreased immune function and more aggressive lymphoma.

MDSCs in Diffuse Large B Cell Lymphoma (DLBCL)

- Patients with DLBCL have higher circulating CD14+ HLA-DR monocytic MDSCs.

MDSCs in Extranodal NK/T Cell Lymphoma

- Study conducted in 32 extranodal NK/T cell lymphoma patients showed higher levels of CD33+ CD11b+ HLA-DR– MDSCs.
- These MDSCs had increased expression of IL-17, arginase-1 and cytokine-inducible nitric oxide synthase (iNOS) and suppressed T cell proliferation.

MDSCs in Cutaneous T Cell Lymphoma

- In cutaneous T cell lymphomas it has been observed that MDSCs and tumor cells inhibit T cell proliferation and promote regulatory FoxP3+ T cells with the expression of programmed death ligand 1 (PD-L1).

Prognosis

- Level of these MDSCs correlates directly with worse clinical prognosis and regulatory T cells (Tregs) proliferation
- The higher levels of MDSCs correlate with shorter progression-free and overall survival.

Research and Studies

- Normal peripheral blood mononuclear cells were incubated with monocytes from patients with B cell non-Hodgkin lymphoma (NHL).
- This resulted in a reduction in T cell proliferation as well as decreased Th1-response observed via measurement of IFN-γ production.
- Anti-CD14 immunomagnetic beads when used to decrease the monocyte population resulted in restored T cell proliferation.

DENDRITIC CELLS

- Dendritic cells aid in the activation of cytotoxic T cells and naive helper T cells.
- Direct follicular dendritic cell contact with the neoplastic cells causes upregulation of microRNA-181a (miR-181a) and reduced levels of proapoptotic Bcl-2-like protein 11 (Bim), resulting in an overall protection from apoptosis.

Dendritic Cells in Follicular Lymphoma

- In follicular lymphoma, tumors with gene expression signatures of dendritic cells and monocytes are shown to be associated with poor outcomes.
- Whereas gene expression signatures of T cell markers and macrophages were associated with prolonged survival.

Research and Studies

- *In vitro* studies were initially promising when DCs were pulsed with either tumor antigen or whole tumor lysate to stimulate immune responses from T cells, however, *in vivo* studies on hematologic malignancies have not demonstrated durable responses.

CHEMOKINES AND CYTOKINES

Role of Chemokines and Cytokines in Classical Hodgkin Lymphoma

- The tumor microenvironment of CHL (constituting 99% of the tumor) is composed of:
  - B cells
  - T cells
  - Eosinophils
  - Plasma cells
  - Neutrophils
  - Macrophages
  - Dendritic cells
  - Fibroblasts.
- All of the above cells are derived from the dysregulated chemokine secretion by the HRS cells.
- Key cytokines playing an active role in the process include:
  - IL-7
  - IL-10
  - TGF-β
  - Chemokine ligand 5 (CCL 5)
  - Chemokine ligand 1 (CCL1)
  - Galectin-1
- T cells surrounding Reed-Sternberg cells express CCL5, which acts as a chemo-attractant for monocytes, eosinophils, basophils and mast cells as well as CD4 positive T cells.
- C-C chemokine receptor type 3 (CCR3) + Th2 cells and eosinophils are attracted by the CCL1(eotaxin) produced by fibroblasts surrounding RS cells.

CYTOTOXIC T CELLS (CTLs)

- During chronic antigen stimulation, lymphocyte activation gene-3 (LAG-3) protein is upregulated on T cells which suppresses CD4+ T cell expansion in response to antigen as well as CD8+ T cell function.
- Specifically, LAG-3 has been shown to maintain tolerance to tumor antigens via its effects on CD8+ T cells.
- In murine models, LAG-3 blockade increases proliferation and effector function of antigen-specific CD8+ T cells within organs and tumors that express their cognate antigen.
- The cytotoxic activity of T cells is enhanced by the targeting of the PD-1 pathway, which can lead to tumor cell lysis.
- Tumor specific activated T cells as well as regulatory T cells express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which binds to CD80/CD86 on antigen

presenting cells and leads to T cell anergy by competing with CD28 as a costimulatory molecule.

CTLs in Lymphoma

- Increased numbers of infiltrating CD8 positive T cells, many expressing cytotoxic markers like TIA-1 have been associated with better outcomes in B cell lymphomas.
- Elevated numbers of cytotoxic lymphocytes positive for programmed cell death-1 (PD-1) was also found to be associated with favorable prognosis in the setting of follicular lymphoma.

REGULATORY T CELLS (TREGs)

- Thymic derived Tregs are involved in preventing autoimmunity.
- Peripheral Tregs maintain tolerance in mucosal sites.
- Both the naturally occurring CD25+ CD4+ Treg populations express FoxP3, which is a more specific marker for regulatory T cells than CD25, CD45RB, or CTLA-4.
- Tregs suppress the activity of bystander T cells, natural killer cells and B cells via CTLA-4, IL-10, and TGF-β1.

Tregs in Inflammation

- FoxP3+ Tregs, particularly in inflamed tissues, express T cell immunoglobulin and mucin-domain containing-3 (TIM-3) which enhances their regulatory function.
- TIM- 3 works as a co-inhibitory receptor that is also expressed on IFN-γ producing T cells, macrophages and dendritic cells, where it leads to inhibition of normal Th1 responses.
- Blockade of TIM-3 signaling appears to demonstrate therapeutic benefit in preclinical tumor models.

Tregs in Lymphoma

- In certain NHL where Tregs are overrepresented in biopsy specimens compared to normal lymphoid tissue, these cells appeared to be recruited by malignant B cells.
- In a study of 280 CHL patients, higher numbers of intratumoral Tregs were associated with better failure free survival and also somewhat better overall survival.
- Similarly, in follicular lymphoma and germinal center subtype diffuse large B cell lymphomas, a positive correlation between disease-specific survival and numbers of intratumoral FoxP3 positive cells was observed.

FOLLICULAR B HELPER T CELLS (T_{FH})

- In the normal germinal center, T_{FH} cells appear to be involved in CD40-mediated interactions in the germinal center.
- Circulating *CD4+ C-X-C chemokine receptor type 5 (CXCR5) + T cells* serve as the memory compartment of T_{FH} cells.
- CXCR5 is the receptor for chemokine ligand 13 (CXCL13), produced by follicular dendritic cells which promotes the entry of B cells into germinal center.
- Hence, the upregulated expression of CXCR5 facilitates contact between the B cells and T cells

T_{FH} in Follicular Lymphoma

- T_{FH} cells are abundant in follicular lymphomas.
- In follicular lymphoma, these cells appear to provide IL-4 stimulation to the B cells and in conjunction with CD40 interactions, aid in the proliferation of neoplastic cells through STAT5 signaling.

T_{FH} in Other Lymphomas

- In patients with low-grade B cell lymphomas like follicular lymphoma or marginal zone lymphomas, circulating T_{FH} cells show reduced C-C chemokine receptor type 6 (CCR6) and increased PD-1.
- Increased levels of PD-1 receptor have also been found in T cells from chronic lymphocytic leukemia (CLL) patients.

NATURAL KILLER (NK) CELLS

- NK cells are CD16+ CD56+ cytotoxic lymphocytes of the innate immune system, which induce apoptosis even in the absence of antibodies and major histocompatibility complex.
- NK cells can recognize tumor antigens via killer-cell immunoglobulin-like receptors (KIRs).
- A subset of NK cells produces IFN-γ, TNF-α, IL-10, and certain chemokines that aid in the differentiation of T cells and dendritic cells.
- Once a tumor microenvironment is developed, TGF-β is induced and TIM-3 expression on NK cells is upregulated.
- Increased TIM-3 expression has been associated with lower NK-cell cytotoxicity and poor outcomes in a variety of neoplasms.

Role of NK Cells in Lymphoma and Therapeutic Potential

- Recent studies have shown significant reduction in NKG2D expression as well as weak cytotoxic activity in NK cells in untreated CHL patients.
- Reactivation of silenced NK cells in CHL is a potential therapeutic target and is being currently pursued. Immune checkpoint inhibitors, like Nivolumab, are being used to recover cytotoxic activity of NK cells in CHL by PD-1 inhibition.
- Drugs targeting heat shock protein-90 have been found to be effective in preclinical studies.

Newer Findings

- In a recent phase 1 study, the bispecific (CD30/CD16a), tetravalent antibody, AFM13 has proven significantly effective in NK cell activation.
- Studies have shown defective NK cell cytolytic function in CLL which is associated with increased cancer risk.
- In mouse models, IFN-γ and perforin protein knockouts develop B cell lymphomas that show suggestion of immunosurveillance defect.

BYSTANDER B CELLS

- Bystander CD 20+ B cells are more numerous in lymphocyte predominant Hodgkin lymphoma (LP-HL) compared with CHL.
- B cell production of IL-10 may aid in antitumor immunosuppression by T cell inhibition.
- Whereas competition with tumor cells (H-RS) for T cell derived survival signals may halt tumor cell growth.

LYMPHOMA EXOSOME

- Exosomes are microparticles that can be secreted by cells and usually range in size from 30 to 100 nm.
- These were thought to be cellular waste, but are now known as carriers of signaling molecules.
- CLL-derived exosomes were shown to induce stromal cells to take on a cancer-associated fibroblast (CAF) phenotype *in vitro*.
- The CAFs, in turn, support a niche that promotes CLL cell adhesion, survival and growth *in vivo*.
- Exosomes produced by lymphoma B cells carrying mutated *MYD88* were reported to reprogram the marrow microenvironment such that mast cells and macrophages were induced to promote endogenous proinflammatory signaling pathways.

EXTRACELLULAR MATRIX (ECM)

- Extracellular matrix facilitates the creation of tumorigenic microenvironment by promotion of angiogenesis and inflammation.
- In solid organ tumors, dysregulated ECM has been shown to expedite cancer progression directly by the following mechanisms:
  - Directly affecting cancer cells causing cellular transformation.
  - Cancer stem cell expansion and
  - Disruption of tissue polarity leading to tumor invasion and metastasis.

ANGIOGENESIS

- Lymphoma tumor microenvironment also includes a rich scaffold of vessels that supply nutrients to the proliferating cells.
- Platelet-derived growth factor (PDGF) recruits PDGF receptor-expressing pericytes to neovessels, thus promoting vascular maturation and stabilization.
- PDGF is also involved in the expression of other stromal angiogenic factors like basic fibroblast growth factor and VEGF.
- Inhibition of platelet-derived growth factor receptor B (PDGFRB) with imatinib mesylate or sunitinib malate has shown some efficacy in carcinoma models.
- One study showed impaired growth of lymphoma in both human xenograft and mouse allograft models with the use of imatinib, a tyrosine kinase inhibitor of PDGFRB.
- Imatinib induces apoptosis of tumor associated PDGFRB positive pericytes and loss of perivascular integrity.

- The tumor endothelium has also been shown to prevent T cell homing, and hence, can serve as a barrier against immunotherapy.
- An inhibitor for endothelin B receptor was shown to increase the adhesion of T cells *in vitro* to human endothelium.
- Inhibitory enzymes, like indolediamine oxidase (IDO), and phenylalanine oxidase interleukin 4-induced gene 1 (IL4I1).
- IL4, secreted by lymphoma associated macrophages and some B-NHL cells
- Immune suppression by Treg expansion and inhibition of effector T cell proliferation and activity.
- Increased expression of FAS ligand (FASL) by NHL B cell induces cytotoxic T cell apoptosis, whereas
- IL-12 secretion induces T cell exhaustion by LAG-3 and TIM-3 induction immunotherapy.

MECHANISMS OF TUMOR MICROENVIRONMENT MEDIATED IMMUNE EVASION AND TUMOR PROGRESSION IN NHL

- A number of mechanisms are implicated in tumor immune evasion and progression.
  - Reduced tumor immunogenicity and immune evasion
  - Loss of lymphoma cell surface molecules/ markers
  - Genetic alterations leading to loss of MHC class I, MHC class II, and CD58 contribute to the failure of CD8+ T lymphocyte, CD4+ lymphocyte, and NK cell-mediated tumor cytotoxicity
  - Overexpression of inhibitory lymphoma cell surface molecules, like PD-L1 and herpesvirus entry mediator (HVEM), which on interaction with their counterparts on T cells lead to impaired T/NK cell activity.
  - Overexpression of CD47 and SIRP-alpha is a lymphoma cell mechanism to evade macrophage-mediated destruction.

Types of Immunotherapy

- Monoclonal antibodies
- Immunomodulating drugs
- Immune checkpoint inhibitors
- CAR T cell therapy.

Monoclonal Antibodies

- They keep cancer cells from growing by blocking signals sent out by the cancer cells.
- They can also bind to the cancer cells and trigger the immune system to kill them.
- Monoclonal antibodies can be attached to toxins, chemo, or radioactive substances.
- Examples:
  - Rituximab: Anti-CD20
  - Brentuximab: Anti-CD30
  - Alemtuzumab: Anti-CD52
- **Follicular lymphoma**
  - Rituximab + chemo
- **Mantle cell lymphomas**
  - Rituximab for maintenance, induction and relapse.

- **Diffuse large B cell lymphoma**
  - Rituximab
  - For relapse: Pembrolizumab
- **T cell Lymphoma**
  - If non-responsive to chemo alemtuzumab/brentuximab.

CHIMERIC ANTIGEN RECEPTOR CAR-T CELL THERAPY

- Immune cells called T cells are removed from the patient's blood.
- They are altered in the lab to have specific receptors (called *chimeric antigen receptors*, or CARs) on their surface.
- These receptors can attach to proteins on the surface of lymphoma cells.
- The T cells are then multiplied in the lab and given back into the patient's blood, where they can seek out the lymphoma cells and launch a precise immune attack against them.
- **Most CAR T cell therapies are still being studied and only available in clinical trials.**
- **Axicabtagene ciloleucel (Yescarta)** approved by the USFDA for:
  - Diffuse large B cell lymphoma
  - Primary mediastinal large B cell lymphoma
  - High grade B cell lymphoma
  - Diffuse large B cell lymphoma arising from follicular lymphoma
- **Tisagenlecleucel (Kymriah)**
  - Diffuse large B cell lymphoma
  - High grade B cell lymphoma
  - Diffuse large B cell lymphoma arising from follicular lymphoma.

POINTS TO REMEMBER

- Composition of the microenvironment plays a vital role in various processes, including the progression, drug resistance and prognosis of lymphoma.
- Targeting microenvironment components is expected to provide novel insights for the precise treatment of lymphoma.
- Nevertheless, there are still many unresolved issues, such as safety, efficacy, drug resistance and the feasibility of drug combination.
- Further studies are warranted to verify and promote the clinical applications of microenvironment-based targeted therapy.
- A deeper understanding of the contribution of microenvironment to lymphomas will help us provide patients with more feasible and effective treatment strategies.

BIBLIOGRAPHY

1. Bejarano L, Jordão M, Joyce J. Therapeutic targeting of the tumor microenvironment. Cancer Discov. 2021;11(4):933–59.
2. Casey S, Amedei A, Aquilano K, Azmi A, Benencia F, Bhakta D, et al. Cancer prevention and therapy through the modulation of the tumor microenvironment. Semin Cancer Biol. 2015;8:S199–223.

3. Ennishi D, Hsi ED, Steidl C, Scott DW. Toward a new molecular taxonomy of diffuse large B cell lymphoma. Cancer Discov. 2020;10(9):1267–81.
4. Hui L, Chen Y. Tumor microenvironment: sanctuary of the devil. Cancer Lett. 2015;368(1):7–13.
5. Junttila M, de Sauvage F. Influence of tumour micro-environment heterogeneity on therapeutic response. Nature. 2013;501(7467):346–54.
6. Sehn LH, Salles G. Diffuse large B cell lymphoma. N Engl J Med. 2021;384(9):842–58.
7. Steidl C, Lee T, Shah S, Farinha P, Han G, Nayar T, et al. Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med. 2010;362(10):875–85.
8. Taskinen M, Karjalainen-Lindsberg M, Nyman H, Eerola L, Leppa S. A high tumor-associated macrophage content predicts favorable outcome in follicular lymphoma patients treated with rituximab and cyclophosphamide-doxorubicin-vincristine-prednisone. Clin Cancer Res. 2007;13(19):5784–9.
9. Wang L, Ding K, Zheng C, Xiao H, Liu X, Sun L, et al. Detachable nanoparticle-enhanced chemoimmunotherapy based on precise killing of tumor seeds and normalizing the growing soil strategy. Nano Lett. 2020;20(9):6272–80.
10. Wu K, Lin K, Li X, Yuan X, Xu P, Ni P, et al. Redefining tumor-associated macrophage subpopulations and functions in the tumor microenvironment. Front Immunol. 2020;11:1731.

PD-1/PD-L1 Pathway in Carcinogenesis

9

Naveen Kumar

INTRODUCTION

Programmed Cell Death Protein 1/ CD279.

- The PD-1 protein in humans is encoded by the PDCD1 gene.
- It is expressed on T cells, B cells, monocytes, natural killer cells, dendritic cells and many tumor-infiltrating lymphocytes (TILs).
- It plays an important role in downregulating the immune system and promoting self-tolerance by suppressing T cell activity.
- PD-1 binds two ligands, PD-L1 and PD-L2.
- PD-L1 (CD274) and PD-L2 (CD273) are both coinhibitory.
- PD-L1 is expressed on resting T cells, B cells, dendritic cells, macrophage, vascular endothelial cells and pancreatic islet cells.
- PD-L2 expression is seen on macrophages and dendritic cells alone.
- PD-1 is an immune checkpoint.
- PD-1/PD-L1 interaction ensures that the immune system is activated only at the appropriate time in order to minimize the possibility of chronic autoimmune inflammation.
- It guards against autoimmunity through a dual mechanism of promoting apoptosis (programmed cell death) in antigen-specific T cells in lymph nodes while simultaneously reducing apoptosis in regulatory T cells (anti-inflammatory, suppressive T cells).

MECHANISM OF PD-1/PD-L1 SIGNALING

- In the tumor microenvironment, PD-1 and its ligand PD-L1 perform a vital role in tumor progression and survival by escaping tumor neutralizing immune surveillance.
- Engagement of PD-L1 with PD-1 of T cell creates T cell dysfunction, exhaustion, neutralization, and production in a tumor mass.
- B7-1 (CD80) is a protein expressed on activated T cells and APCs which interacts with the PD-L1 of tumor cells.
- This causes negative regulation of effector T cell activation.
- The function of a tumor overexpressing PD-L1 is to protect itself from cytotoxic T cell mediated cell killing.

- Another subtype of T cells, such as regulatory T cells (Treg, CD4+) create a highly immunosuppressive tumor environment.
- This is done by maintaining the expression of PD-1 on cell surface.
- PD-1 increases the *de novo* transformation of naive CD4+ T cells to Treg cells, thus attenuating immune responses.
- PD-L1 is expressed in a wide range of hematopoietic and non-hematopoietic cells.
- PD-L2 has restricted expression on macrophages, dendritic cells (DCs) and mast cells in the secretion of IL-4 and IFN–γ.
- It has been recently reported that PD-L2 interacts with repulsive guidance molecule B (RGMB) of macrophage (MΦ) proteins.
- Although, there are several reports on PD-L2, little information is available about its role in cancer immunosuppression.
- PD-L1 is responsible for tumor immune modulation.
- The binding affinity of PD-1 with PD-L1 is three times greater than the affinity between PD-1 for PD-L2.
- PD-L1 expressions in tumor cells and hematopoietic cells are determined by the stimulation of pro-inflammatory cytokines such as IFN-γ and TNF-α.
- PD-L1 of tumor stromal components, such as fibroblast, extracellular matrix (ECM), tumor-associated macrophages (TAM), and myeloid derived suppressor cells (MDSC) deactivate T cell (CD8+) mediated cancer cells killing through interaction with PD-1.
- Similarly, maturation of MDSC to TAM and secretion of pro-inflammatory cytokines (IFN-γ) from TAM suppress T cell functions.
- This provides positive modulation of PD-1 and PD-L1 interaction.

Mechanism of PD-1/PD-L1 Mediated Immune Resistance

- PD-1-associated immune-resistance depends on the accessibility of PD-L1 ligand in the tumor.
- There are two types of immune resistance:
  1. Innate immune resistance
  2. Adaptive immune resistance

Innate Immune Resistance

- In glioblastomas PD-L1 expression is by downregulation of PTEN which is linked to activation of PI3K-Akt tumorigenic signaling.
- In lymphomas and lung cancers PD-L1 expression occurs through upregulation of the STAT3 and lymphoma kinase (ALK) signaling resistance.
- The STAT3 activation is modulated through IL-6 and the IL-6-STAT3 axis is considered as one of the crucial pathways in tumorigenic immune suppression.

Adaptive Immune Resistance

- PD-L1 expression is induced due to the secretion of pro-inflammatory IFN-γ from tumor and tumor-stromal cells that neutralize CD8+ cytotoxic T cell-induced anti-tumor immune responses.
- Brandon et al investigated the clinicopathologic characteristics of non-small cell lung cancer (NSCLC) subsets defined by PD-L1 expression in either tumor cells or tumor-infiltrating immune cells.

- PD-L1 expression by IHC is currently the only available predictive biomarker for NSCLC response to immune checkpoint blockade.
- PD-L1 expression in either tumor cells or tumor-infiltrating immune cells is correlated with high histologic grade and solid subtype.

PD-1/PD-L1 in Hematological Malignancies

- Among hematological malignancies, PD-1/PD-L1 inhibitors have been successful, so far, only in the treatment of classical Hodgkin lymphoma.
- Typically exhibits an over-expression of PD-1 ligands.
- It is due to alterations in chromosome 9p24.1 because the locus contains the genes encoding PD-L1 and JAK2.
- PD-L1 and PD-L2 are expressed on the surface of malignant cells in 65–100% of classical Hodgkin's lymphoma and in 54% of nodular lymphocyte predominant Hodgkin's lymphoma.
- This results in increased expression of proteins on RS cells.
- JAK2–STAT signaling promotes further increased expression of PD-L1 by augmenting transcription of the PD-L1 gene.

STRATEGIES TO MEASURE PD-L1/PD-1 EXPRESSION

- Immunohistochemistry is one potential biomarker, however, yet to be standardized.
- Challenges regarding standardization are as follows:
  - Use of different monoclonal antibody clones.
  - Heterogeneous PD-L1 expression in different regions of the same tumor specimen.
  - Absence of PD-L1 expression on small biopsies may not reflect the systemic immunologic landscape.
- Other methods like flow cytometry which has an added advantage of simultaneously measuring the expression of PD-1 and PD-L1 in malignant cells and immune cells.
- Immunomagnetic selection and cell track analyzer.
- ELISA, FISH probe specifically targeting the gene locus encoding PD-L1 located at 9p24.1
- The incidence of 9p24.1 amplification (at least >3 copies) is seen in early-stage HL-24% and in advanced stage HL-50%.
- Patients with HL harboring 9p24.1 amplifications had a shorter progression-free survival.
- FISH studies promise to be another potential method for predicting the response to PD-1/PD-L1 treatment.

PD-1/PD-L1 Inhibition and Uses

- Monoclonal antibodies are drugs called checkpoint inhibitors.
- They reduce toxicity within tolerable limits.
- Shrink solid tumors.
- Suppress advanced tumors and metastasis.
- Overall improve patient survival.
- Inhibitors will cause resurrection of T cell mediated anti-tumor immune effect.

Targeted Therapy

- **PD-1 inhibitors:** Nivolumab and Pembrolizumab.
- Nivolumab has been approved by USFDA in treatment of advanced and metastatic cases of melanoma, head and neck squamous cell carcinoma, non-small cell lung cancer and renal cell carcinoma.
- Pembrolizumab has been approved by USFDA for treatment of recurrent and metastatic cases of head and neck squamous cell carcinoma.

PD-L1 Inhibitor

- Atezolizumab has been approved by USFDA in the treatment of non-small cell lung cancer and in the treatment of urothelial carcinoma.

PD-1 INHIBITORS FOR COMBINATION THERAPY

- Monotherapeutics have been demonstrated to be a successful immunotherapy regimen.
- Because of safety and better clinical activity of monotherapy the field is moving toward the direction of discovering novel combination therapies.
- Various anti-cancer agents are used in combination with PD-1 antibody inhibitors.

Combination of PD-1 and Kinases Inhibitors

- Mitogen-activated protein kinase is an effective regulator of BRAF mutation which is responsible for the metastasis of cutaneous melanoma.
- The mutation of BRAF has also increased the expression of the PD-1 and PDL-1 molecule.
- It induces potential drug resistance with the involvement of tumor stromal cells.
- The combination of PD1 immunotherapy with a BRAF inhibitor resulted in synergistic anti-tumor response and prominent tumor growth inhibition.

Combination of PD-1 and Other Checkpoint Inhibitors

- For example, Nivolumab and Anti-CTLA-4 antibody- ipilimumab are under clinical trials in treatment of various cancers.
- Various other combinations of PD-1 with VEGF inhibitors and chemotherapeutic drugs are under clinical trials.

Adverse Reactions

- Once the immune checkpoints have been blocked, the equilibrium between the autoimmunity and immune tolerance will be affected.
- Immune-meditated adverse reactions (IMARs) is a term coined to describe the side effects of new immunotherapy.
- Adverse effects like fatigue (32%), rash (23%), skin disorders (36%), GI events (18%), endocrinopathies (13%) and diarrhea (18%).

POINTS TO REMEMBER

- Immune checkpoint mechanisms such as the PD-1/PD-L1 pathway have led to a clinically significant antitumor response.

- Rapid improvement is being demonstrated in the treatment of various cancers which resulted in USFDA approval for these newly discovered agents.
- Currently, multiple studies are undergoing to improve the success rate and more efficient use of these agents at an earlier stage of the disease in single or in combination.
- This can significantly increase overall survival or cure rates.

BIBLIOGRAPHY

1. Alsaab HO, Sau S, Alzhrani R, et al. PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. Front Pharmacol. 2017;8:561.
2. Butte MJ, Keir ME, Phamduy TB, Freeman GJ, Sharpe AH. PD-L1 interacts specifically with B7–1 to inhibit T cell proliferation. Immunity. 2009;27:111–22.
3. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. Immunol Rev. 2010;236: 219–42.
4. Martin AM, Nirschl TR, Nirschl CJ, Francica BJ, Kochel CM, van Bokhoven A, et al. Paucity of PD-L1 expression in prostate cancer: innate and adaptive immune resistance. Prostate Cancer Prostatic Dis. 2015;18: 325–32.
5. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012; 12:252–64.
6. Ribas A. Adaptive immune resistance: how cancer protects from immune attack. Cancer Discov. 2015;10:915–9.
7. Sau S, Banerjee R. Cationic lipid-conjugated dexamethasone as a selective antitumor agent. Eur J Med Chem 2014;083:433–47.
8. Sun Z, Fourcade J, Pagliano O, Chauvin JM, Sander C, Kirkwood JM. IL10 and PD-1 cooperate to limit the activity of tumor-specific CD8+ T cells. Cancer Res. 2015;75: 1635–44.
9. Turley SJ, Cremasco V, Astarita JL. Immunological hallmarks of stromal cells in the tumour microenvironment. Nat Rev Immunol. 2015;15:669–82.
10. Xiao Y., Yu S., Zhu B., Bedoret D., Bu X., Francisco L. M., et al. RGMB is a novel binding partner for PD-L2 and its engagement with PD-L2 promotes respiratory tolerance. J Exp Med. 2014; 211:943–959.

10 Techniques to Characterize Foreign Materials in Pathological Specimens

Naveen Kumar

INTRODUCTION

- Pathologists typically encounter many exogenous materials in clinical specimens during routine histopathological examinations.

Foreign Substances

- **Voluntary causes** (e.g. tattoos or cosmetic fillers)
- **Involuntary causes** (e.g. trauma, surgery, and ingestion or inhalation of particulate materials).
- Most are incidental findings seen in histological or cytological material removed for other purpose (e.g. dermal suture granulomas, lubricant in Pap smears).
- Some are removed because they are the cause of an undiagnosed lesion (e.g. dental amalgam tattoo mimicking malignant melanoma).
- They are easily recognized by routine light microscopy, and do not pose a diagnostic problem.
- However, on occasion a full characterization becomes important to rule out other entities (e.g. melanin vs. dental amalgam), and confirm the diagnosis (e.g. cutaneous deposits of silver in argyria).
- Histopathologically, they generate immune, inflammatory, or granulomatous reactions.
- Microscopic examination is not always sufficient to identify the nature of these foreign substances on the basis of characteristic appearance and birefringence patterns.
- They are usually described as particles, pigments, granules, fibers, or fragments of various forms, sizes, and colors.

FOREIGN MATERIALS

Tattoos

- Mechanical introduction of insoluble pigments into the dermis.
- With the increasing incidence of tattooing fashion trend, physicians should be able to recognize tattoo complications.

Tattoo Reactions

- **Inflammatory:** Allergic to salts used, focal oedema, pruritus, papules, or nodules at the tattoo site.
- **Infections:** Bacterial, viral or mycotic.
- **Neoplastic complication** rarely described within tattoo pigments includes keratoacanthoma, squamous cell and basal cell carcinoma, leiomyosarcoma, and melanoma.
- **Microscopically** they localize around vessels in the upper and mid-dermis in macrophages and fibroblasts (Fig. 10.1).
- **Extracellular deposits:** Found between collagen bundles. The pigment is generally refractile but not doubly refractile.

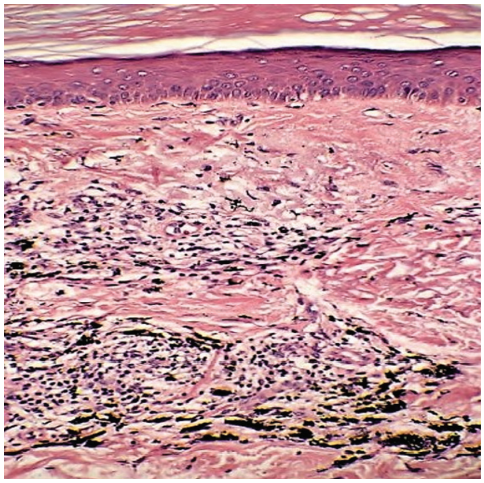

Fig. 10.1: Tattoo pigment with associated inflammatory reaction

Suture

Various types of suture materials are as follows:

- **Common:** Silk, polyvinylidene fluoride (PVDF), polyglycolic acid, and catgut.
- **Absorbable:** Catgut, polydioxanone, polyglycolic acid. They are used for deep tissues, membranes, and subcuticular skin closure.
- **Non-absorbable:** Polypropylene, nylon (Fig. 10.2), stainless steel. They are used for skin (removed) and some deep structures (tendons, vessels, nerve repairs—not removed).

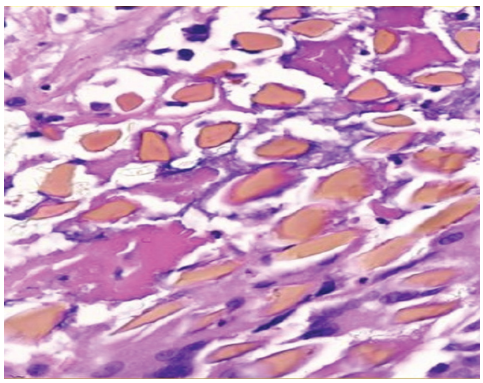

Fig. 10.2: Nylon suture granuloma

Polypropylene

- These are non-absorbable meshes for hernia repair and emergency abdominal wall reconstruction.
- They are colorless rounded structures on H&E and brightly birefringent on polarized light.[2]

Silicone

- Silicone is an organosilicon compound which can be in liquid, gel or solid form.
- A liquid form of silicone is dimethicone (dimethylpolysiloxane).
- **On H&E** they are refractile, colorless, non-birefringent and usually found within phagocyte vacuoles or extracellularly, especially lining partially "washed-out" spaces.

Collagen Implant

- **Natural polymers:** Bovine collagen and nonsulfated glycosaminoglycan, hyaluronic acid.
- The injection of bovine collagen is a relatively safe procedure used to correct defects caused by acne scars, trauma, and aging.

Adverse Reactions

- Erythema
- Urticaria
- Abscess formation
- Induration of the injection site
- Granulomatous reactions.
- There is a theoretical risk of prion transmission when materials of bovine origin are used.
- No such complication has yet been reported.
- On microscopy they are acellular and structureless with diffuse eosinophilic lobules.
- Bovine collagen is non-refringent and stains a pale gray-violet color with Masson's trichrome stain.
- It is absorbed and it can no longer be detected by light microscopy or immunofluorescence techniques after several months.
- The native collagen is different from bovine collagen because it is birefringent and stains green with Masson's trichrome stain.

Hyaluronate

- They present as diffuse nodular lesions in the labial vestibule.
- Represent as hyaluronate dermal fill granulomas that migrated from the original injection sites.
- They are positive for alcian blue and mucicarmine.

Synthetic Fillers

Lactate Polymer

- They are unique and form foreign body granuloma surrounding ***"surf board" shaped large vacuoles that contain "broken glass-like particles".***

Hydroxyethyl Methacrylate

- They form well circumscribed granuloma and show stretched multinucleated giant cells.
- The cleft contents of hydroxyethyl methacrylate are **nonrefractile.**

Cellulose

- Cellulose is present in tissues as cotton, wood splinter, food particles (aspiration), IVDU (microcrystalline cellulose from oral medications).
- It does not stain well with H&E but it is birefringent under polarized light.
- Special stains (GMS, PAS) are also helpful in identification.

Dental Amalgam

- Dental amalgam has multiphasic material containing silver (Ag), tin (Sn), mercury (Hg), and lesser amounts of copper (Cu).
- Incidental tattooing of buccal mucosa may occur during dental procedures (Fig. 10.3).
- Prolonged tissue implantation leads to loss of mercury and tin, and persistence of silver with sulfur (S) and selenium (Se) deposition.

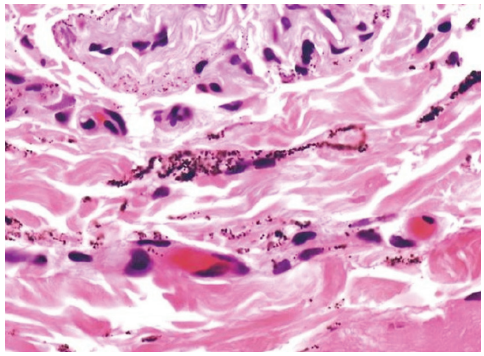

Fig 10.3: Dental amalgam tattoo pigment in sub-epithelium of buccal mucosa

Starch

- Starch powder, a lubricant of surgical gloves, is well recognized as a common contaminant.
- They are refractile, glassy, polygonal bodies, 5–20 micrometer in diameter.
- They often exhibit a ***central dot or "Y"-shaped structure.***
- The ***Maltese cross under polarized light is characteristic,*** but not specific, for starch and can also be seen with some inorganic particles.

Talc

- Talc or hydrated magnesium silicate, is formed during the breakdown of anthrophyllite rock.
- It is used in gloves and as a binder in oral tablets to hold the medication together.
- It is polarizable and birefringent foreign body.

Acrylic Polyamide Plastic Embolization Material

- Embolization microspheres have been developed for tumor embolization and treatment of vascular malformations.
- Uterine artery embolization for treatment of fibroids.
- Several materials have been used like polyvinyl alcohol, collagen, dextran.
- On microscopy they are rounded often folded circular eosinophilic to weakly basophilic objects usually in an intravascular location (Fig. 10.4).
- It is usually mistaken for parasite.
- Mucicarmine, trichrome and Sirius red stains are positive.

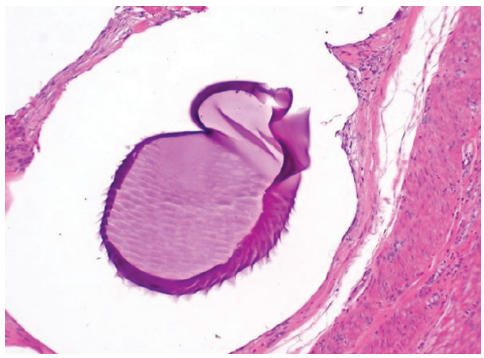

Fig. 10.4: Intravascular acrylic polyamide plastic with Venetian blind artifact mimicking parasite

DRUG DEPOSITS AND PIGMENTATION

Antimalarial Drugs

- The long-term use causes cutaneous pigmentation.
- Yellow pigmentation seen with quinacrine (mepacrine), although the histopathology has not been described.

- Pretibial pigmentation is more common—slate gray to blue-black in color.
- Pigment granules have variable staining characteristics like some staining for hemosiderin, some for melanin, and some for both.
- They can be seen in macrophages as well as extracellularly.

Phenothiazines

- It causes gray-blue pigmentation in sun-exposed areas.
- On microscopy, refractile, golden-brown pigment with staining properties of melanin is found in the dermis along collagen bundles.
- It is seen in macrophages, especially around vessels.
- Electron microscopy shows melanin granules in macrophages and other bodies of varying electron densities that represents metabolites or complexes of the drug.

Tetracycline

- Causes teeth pigmentation.
- Bluish pigmentation of cutaneous osteomas.
- Bluish-green pigmentation in areas of trauma on the lower legs, has resulted from the use of tetracycline.
- Rarely, pigmentation of acne scars on the face occurs.

Minocycline

- Bluish-black pigmentation of scars.
- Blue-gray circumscribed pigmentation of the lower legs and arms.
- They are present in macrophages, perivascular areas and dermal dendrocytes.
- May deposit on elastic fibers or lie free in the dermis.
- Pigment is positive with both the Perls' method for iron and the Masson–Fontana method for melanin.
- It is non-birefringent and nonfluorescent.

Amiodarone

- Slate-gray discoloration of sun-exposed areas.
- On H&E, yellow-brown granules are found in macrophages, which tend to accumulate around blood vessels at the junction of the papillary and reticular dermis.
- Generally believed to be lipofuscin but recent evidence suggest that the material represents amiodarone itself.
- The granules stain positively with the PAS, prolonged Ziehl–Neelsen, Fontana, and Sudan black methods.

OTHER METHODS

- Infrared spectroscopy
- Raman laser spectroscopy
- Scanning electron microscopy with energy dispersive X-ray analysis.

Recent Advance

- Laser-induced breakdown spectroscopy.

Scanning Electron Microscopy with Energy Dispersive X-ray Analysis (SEM/EDXA):

- It was first introduced in the 1960s.
- It has both scanning electron microscopy (SEM) together with energy dispersing microanalyzer (EDX).
- It was used by Raso et al, to examine the bioreactivity of silicone implants.
- It is a method for determining the elemental composition of a particle that can be localized in a tissue section.
- SEM together with EDX are used in toxicology and pathology for determining exogenic and endogenic toxic substances.
- Generally, provides qualitative information.
- Quantitative SEM/EDXA are available particularly for materials for which laboratory standards of known composition are available.

Sample Processing

- Routinely an unstained section of 5 μm on a carbon disc and place the adjacent section from the paraffin block on a glass slide for H&E staining.
- Localize area of interest on H&E and compare to the carbon disc.
- If only an original stained section is available on glass, SEM/EDXA can still be useful.
- Remove coverslip and the background glass examined away from the specimen, will contain elements in glass (usually Si, O, Ca). (Na, Mg, Al, Cl, K, variably present).

Infrared Spectrophotometry

- It became available in 1940s and gives a molecular "fingerprint" that can be compared to reference spectra.
- Unstained section of 5 μm adjacent to H&E stained section or carbon disc section placed on aluminum coated (reflective) glass slide can be used for this.
- Principle –
  Energy of molecule = Electronic energy+ Vibrational energy + Rotational energy.
- When energy in the form of infrared radiation is applied then it causes the vibration between the atoms of the molecules.
- When the applied infrared frequency is equal to natural frequency of vibration then Absorption of IR radiation takes place and a peak is observed.
- Different functional groups absorb characteristic frequencies of IR radiation.
- It gives the characteristic peak value.
- IR spectrum of a chemical substance is a **finger print of a molecule for its identification.**
- IR spectroscopy is concerned with the study of absorption of infrared radiation, which causes vibrational transition in the molecule.
- It is ***Also known as Vibrational spectroscopy.***

Raman Spectroscopy

- Sir Chandrasekhara Venkata Raman who won the Nobel prize in Physics for discovering the 'Raman effect'.
- **Principle:** When monochromatic radiation is incident upon a sample then this light will interact with the sample.
- It may be reflected, absorbed or scattered in some manner.

- It is the scattering of the radiation that occurs which gives information about molecular structure.
- Raman effect is based on scattering.

Recent Advance

Laser-induced Breakdown Spectroscopy (LIBS)

- An all-optical instrument based on laser-induced breakdown spectroscopy (LIBS) has been developed to create multi-elemental images of biological tissues.
- It was primarily developed to visualize the distribution and kinetics of metal nanoparticles in the kidneys of laboratory animals.
- LIBS imaging successfully elucidated the chemical nature and distribution of exogenous agents, such as aluminum (Al) in a granuloma and a pseudolymphoma.
- It also demonstrated Titanium (Ti), copper (Cu), chromium (Cr) and tungsten (W) in lymph nodes and/or the skin.
- **Principle:** A magnification lens is used to focus the laser pulse.
- Then the plasma emission is collected by 2 lenses.
- This is connected to the fiber systems coupled to a spectrometer.
- Spectrometer is equipped with a camera synchronized to the laser.
- It can be performed directly on the paraffin block using Nd:YAG laser.

BACKGROUND ELEMENTS OF TISSUE

- Phosphorus (P) or sodium (Na) is a constitutive element of every cell in tissue.
- This creates elemental images of 'background elements' using LIBS.
- This allows proper visualization of the global architecture of the tissue.

Subcutaneous Nodular Lesions

- Persistent subcutaneous nodular lesions following vaccination or hyposensitization therapy.
- Cutaneous adverse reactions following vaccines or hyposensitization therapy containing Al salts are common.
- Overlapping histopathological patterns mimic the patterns of other conditions such as granuloma, panniculitis, or pseudolymphoma.
- LIBS allows direct *in situ* visualization of the levels of Al in a skin nodule and at the site of hyposensitization therapy.

Pigmented Lymph Nodes

- Pigmentated lymph node is highly suggestive of metastatic malignant melanoma.
- However, exogenous ink particles from tattoos are the most frequent cause.
- This represents a pitfall for surgeons and pathologists during lymph node biopsies or dissection procedures.
- Pigmented lymph nodes pose a diagnostic challenge.
- LIBS has been used to find out the exact element present.
- Elevated levels of titanium (Ti) in lymph nodes that corresponded with the topography of the black pigment reported after histopathological examination. This confirmed the diagnosis of Tattoo pigment.

Role of LIBS

- A need exists for *in situ* elemental imaging of a steadily growing number of identified diseases characterized by a metal imbalance in cells and tissues (e.g. metal overload diseases, neurodegenerative diseases, or even cancers).
- LIBS could provide robust, valuable complementary elemental information to unravel the pathogenesis of such metal-related diseases.
- Combination of standard histochemical procedures with LIBS multielemental imaging is a promising approach for routine investigations of the chemical composition of foreign substances contained in paraffin a embedded specimens.

POINTS TO REMEMBER

- Medical uses of exogenous materials are widespread and will likely increase.
- New medical materials and devices will continue to appear in pathology specimens.
- Familiarity with these materials may help pathologists avoid possible sources of diagnostic error.
- Accurate characterization of these foreign materials can benefit patients, clinicians and regulatory agencies to monitor and change materials that causes harm to patients.

BIBLIOGRAPHY

1. Busser B, Moncayo S, Trichard F, et al. Characterization of foreign materials in paraffin-embedded pathological specimens using *in situ* multi-elemental imaging with laser spectroscopy. Modern Path, 2017.
2. Eversole R, Tran K, Hansen D, Campbell J. Lip augmentation dermal filler reactions, histopathologic features. Head and Neck Pathology. 2013.
3. Gimenez Y, Busser B, Trichard F, et al. 3D imaging of nanoparticle distribution in biological tissue by laser-induced breakdown spectroscopy. Sci Rep. 2016;6:29936.
4. Kakoei S, Baghnei F, Dabiri S, et al. A comparative *in vivo* study of tissue reactions to four suturing materials. IEJ, 2010.
5. McRae R, Bagchi P, Sumalekshmy S, et al. *In situ* imaging of metals in cells and tissues. Chem Rev. 2009;109:4780–4827.
6. Molina-Ruiz AM, Requena L. Foreign body granulomas. Dermatol Clin. 2015;33:497–523.
7. Patterson JW, Hosler GA. In: Weedon's Skin Pathology. 4th Edn, 2016, Edinburgh: Churchill Livingstone/Elsevier.
8. Requena L, Cerroni L, Kutzner H. Histopathologic patterns associated with external agents. Dermatol Clin. 2012;30:731–48 vii.
9. Sancey L, Motto-Ros V, Busser B et al. Laser spectrometry for multi-elemental imaging of biological tissues. Sci Rep. 2014;4:6065.