ImmunoTools *FlowISiAM* Award 2026

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Identification of Antigen-Associated $CD14^{+}CD16^{+}$ Monocyte Surface Signatures as Early Predictive Biomarkers in Multiple Myeloma Progression using *AMANDUS* and *FlowISiAM*

Multiple myeloma (MM) is a high-prevalence hematologic malignancy characterized by progressive immune dysregulation and bone marrow microenvironment remodeling. Although effective therapies exist for newly diagnosed multiple myeloma (NDMM), and increasingly sophisticated treatment strategies are available for relapsed/refractory multiple

myeloma (RRMM), the disease remains incurable and marked by eventual progression and therapeutic resistance. Reliable immune biomarkers that predict transition from precursor stages—monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM)—to overt disease, as well as biomarkers that reflect progression from NDMM to RRMM, remain lacking. Early identification of immune alterations across this continuum would enable improved risk stratification, guide therapeutic decision-making at diagnosis, and potentially identify patients at risk of relapse or resistance, thereby informing timely and stage-appropriate intervention strategies.

This proposal addresses this unmet clinical need by focusing on $CD14^+CD16^+$ inflammatory monocytes as early immune sentinels that reflect and potentially drive microenvironmental transformation. We aim to identify disease- and antigen-associated surface marker signatures on these monocytes using high-dimensional flow cytometry, with downstream translational integration into *AMANDUS*-based epitope identification strategies.

Hypothesis

Early myeloma progression is driven by transcriptional and functional reprogramming of $CD14^+CD16^+$ inflammatory monocytes, which expand during MGUS and SMM and establish a permissive tumor-supportive microenvironment. Their ability to recirculate from the bone marrow to peripheral blood allows these cells to capture and disseminate tumor-derived antigens and immune signatures that reflect disease trajectory.

Study Aim: ***To define phenotypic and tumor antigen–associated surface marker signatures of $CD14^+CD16^+$ monocyte subsets across the myeloma continuum from MGUS → SMM → NDMM → RRMM continuum***

Bone marrow aspirates and matched peripheral blood samples will be collected from healthy controls, MGUS, SMM, NDMM and RRMM patients. Using multiparametric flow cytometry, monocyte subsets will be quantified and characterized for activation, antigen-processing, migration, immune-regulatory, and tumor-associated phenotypes. Markers will include core lineage and activation molecules (CD14, CD16, HLA-DR, CD11b, CD11c), macrophage polarization markers (CD163), and candidate tumor antigen–associated markers identified from the literature and preliminary datasets. ImmunoTools will provide some validated monoclonal antibodies for additional potential markers. By comparing matched bone marrow and peripheral blood samples of different disease stages, this study will determine whether disease-associated immune signatures are preserved in circulation, thereby supporting the feasibility of minimally invasive blood-based biomarker detection. Candidate marker profiles will be correlated with clinical parameters, including plasma cell burden and progression status, to establish their predictive relevance.

Background & Significance

Multiple myeloma (MM) is a common and clinically significant plasma cell malignancy characterized not only by intrinsic genetic abnormalities within malignant plasma cells but also by profound remodeling of the bone marrow immune microenvironment (*Rajkumar, 2020*). Clinically, MM manifests with bone pain, anemia, renal dysfunction, hypercalcemia, and recurrent infections, and despite major therapeutic advances, it remains incurable with inevitable relapse. Although immunomodulatory agents, monoclonal antibodies, bispecific antibodies, and CAR-T cell therapies have substantially extended survival (*Moreau et al., 2021; Tang et al., 2025b*), these strategies are largely deployed at advanced disease stages. Consequently, there remains a critical unmet need to identify early immune alterations that predict progression from precursor states—monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM)—to overt malignancy.

Myeloma progression is increasingly recognized as a co-evolutionary process between malignant plasma cells and the surrounding immune and stromal compartments. Myeloma cells express a broad repertoire of surface and intracellular antigens, including CD38, SLAMF7, and B-cell maturation antigen (BCMA), as well as tumor-associated antigens generated through clonal immunoglobulin production, somatic mutation, aberrant glycosylation, and stress responses within the bone marrow niche. These antigens are captured and processed by antigen-presenting cells such as monocytes and macrophages, thereby shaping adaptive immune responses. Importantly, antigen burden and antigen presentation dynamics evolve during disease progression, accompanied by impaired immune surveillance, immune checkpoint upregulation, and progressive immune tolerance. Understanding how tumor-associated antigens are sensed and reflected within the immune microenvironment—particularly during early disease stages—is essential for developing predictive and preventive strategies.

Emerging evidence demonstrates that non-malignant immune compartments actively drive myeloma progression, therapeutic resistance, and immune evasion. Among these, $CD14^{+}CD16^{+}$ intermediate and non-classical monocytes have gained attention as functionally distinct subsets with inflammatory, immunoregulatory, and tissue-remodeling properties. These cells expand in chronic inflammation and cancer and are increasingly implicated in shaping immunosuppressive tumor niches. In MM, $CD14^{+}CD16^{+}$ monocytes exhibit enhanced osteoclastogenic potential and display disease-stage–specific alterations, including IL21R overexpression that distinguishes active myeloma from precursor conditions (*Bolzoni et al., 2017; Petitprez et al., 2015*). Circulating monocyte populations in MM are skewed toward $CD14^{+}CD16^{+}$ subsets, with expansion correlating positively with disease severity, while classical $CD14^{+}CD16^{-}$ monocytes decline with increasing tumor burden (*Zahran et al., 2021*).

Importantly, $CD14^{+}CD16^{+}$ monocytes are not merely bystanders but active regulators of tumor progression. They produce IL-6, TNF-α, TGF-β, and VEGF—cytokines central to

plasma cell survival, angiogenesis, and immune suppression. Their migratory capacity enables trafficking between bone marrow and peripheral blood, raising the possibility that they function as recirculating immune sentinels capable of acquiring and disseminating tumor-associated antigenic signals. Supporting their clinical relevance, the efficacy of the anti-CD38 monoclonal antibody daratumumab is critically dependent on $CD14^{+}CD16^{+}$ monocytes, with CD16 expression levels and monocyte-to-myeloma ratios influencing therapeutic response (*Storti et al., 2020*). These observations highlight $CD14^{+}CD16^{+}$ monocytes as both functional mediators of disease biology and candidate biomarkers of progression.

In parallel, intrinsic transcriptional programs within plasma cells evolve during myeloma pathogenesis (*Tang et al., 2022*). Our recent work on RUNX1 dynamics in plasma cell differentiation demonstrated that RUNX1 is tightly regulated during normal maturation but becomes dysregulated during malignant transformation (*Tang et al., 2025a*). RUNX1-associated pathways intersect with inflammatory and immune signaling networks, underscoring coordinated crosstalk between malignant plasma cells and the immune microenvironment. Integrating plasma cell–intrinsic transcriptional alterations with immune-centric biomarkers such as $CD14^{+}CD16^{+}$ inflammatory monocytes provides a comprehensive framework for understanding early disease evolution.

Despite these insights, early immune alterations that precede overt progression from MGUS and SMM to symptomatic MM remain poorly defined (*Guadagno et al., 2026; Zhang et al., 2025*). Current risk stratification relies predominantly on plasma cell burden and cytogenetic markers, which do not fully capture microenvironmental dynamics. There is a pressing need for minimally invasive, immune-based biomarkers that reflect early antigen exposure, immune conditioning, and microenvironmental remodeling.

This proposal addresses this critical gap by focusing on $CD14^{+}CD16^{+}$ monocytes as sentinel immune regulators during early myeloma progression. We hypothesize that expansion and phenotypic reprogramming of $CD14^{+}CD16^{+}$ monocytes occur at precursor stages and that disease-associated surface marker signatures in these cells can serve as predictive biomarkers of progression. By leveraging high-dimensional flow cytometric profiling to identify antigen-associated and immunoregulatory surface phenotypes across the MGUS → SMM → NDMM→ RRMM continuum, this study aims to define clinically actionable immune signatures detectable in peripheral blood. Defining early immune remodeling within the myeloma microenvironment will advance understanding of tumor–immune co-evolution, enable improved risk stratification, and provide a translational pathway toward biomarker-driven early intervention strategies in multiple myeloma.

Experimental Approaches

(i) Study Design and Patient Cohorts

This study will be conducted as a cross-sectional translational immunology investigation involving five clinically defined cohorts: healthy age-matched controls, MGUS, SMM, NDMM, and RRMM. Approximately 120 participants will be recruited through

established collaborations with hematology centers, ensuring access to well-characterized patient populations across the full disease continuum from precursor states to advanced therapy-resistant disease. All participants will provide informed consent under approved institutional ethical protocols by Universiti Malaya Research Ethics Committee (UMREC).

Diagnostic classification will follow internationally recognized criteria. MGUS and SMM patients will be further stratified using established progression risk models incorporating serum M-protein levels, bone marrow plasma cell percentage, and serum free light chain ratios. NDMM patients will be categorized according to the International Staging System (ISS) and Revised ISS (R-ISS), which integrate β2-microglobulin, albumin, lactate dehydrogenase levels, and cytogenetic abnormalities. RRMM patients will be classified based on prior lines of therapy, refractoriness to proteasome inhibitors and/or immunomodulatory agents, cytogenetic risk profile, and time to relapse. This structured staging framework enables immune phenotypes to be interpreted within clinically meaningful categories reflecting disease evolution and therapeutic pressure.

For each participant, bone marrow aspirates and matched peripheral blood samples will be collected at diagnosis or at the time of relapse prior to initiation of new therapy. Clinical parameters, including plasma cell burden, serum paraprotein levels, cytogenetic abnormalities, bone disease status, renal function, inflammatory markers, prior treatment exposure, and response history in RRMM cases, will be systematically recorded. This comprehensive dataset will allow immune phenotypes to be analyzed in relation to both disease stage and treatment resistance.

(ii) Flow cytometrical analysis and FlowISiAM

High-dimensional multiparametric flow cytometry will be performed to quantify monocyte subsets and characterize their activation, antigen-processing, migratory, and immunoregulatory phenotypes. Monocytes will be defined within the $CD45^{+}$ leukocyte compartment and stratified into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$) subsets. Surface markers will include lineage-defining molecules, chemokine receptors associated with trafficking, antigen-presentation markers such as HLA-DR, immune checkpoint molecules, co-stimulatory proteins, and macrophage polarization markers including CD14, CD16, HLA-DR, CD11b, CD11c, CD163), and candidate tumor antigen–associated markers. Intracellular staining will be incorporated where appropriate to assess antigen-processing–related signatures and tumor-associated immune conditioning. Some antibody panels will be optimized in collaboration with ImmunoTools to ensure reproducibility and translational applicability.

The primary analytical objective will be to determine whether $CD14^{+}CD16^{+}$ monocyte subsets demonstrate progressive expansion and phenotypic reprogramming across the MGUS → SMM → NDMM → RRMM continuum. Quantitative changes in subset frequency will be evaluated alongside qualitative alterations in immune-regulatory marker expression. Particular emphasis will be placed on identifying phenotypic shifts associated with early progression in MGUS and SMM, as well as additional immune adaptations emerging in

RRMM under therapeutic pressure. Comparisons between bone marrow and peripheral blood compartments will assess whether disease-associated immune signatures remain detectable in circulation, thereby supporting minimally invasive biomarker strategies.

Furthermore, use the ***FlowISiAM*** AT-test (anti-Apo10 and anti-TKTL1) as well as ***FlowISiAM*** cytokine-test for intracellular cytokines like IL-6.

(ii) Correlation study and Statistical analysis

Statistical analyses will establish associations between monocyte phenotypes and clinical staging parameters. Group comparisons across disease categories will be performed using non-parametric methods depending on data distribution. Correlation analyses will evaluate relationships between monocyte marker expression and plasma cell burden, M-protein levels, β2-microglobulin concentrations, free light chain ratios, ISS/R-ISS stage, and number of prior therapy lines in RRMM. Multivariate regression modeling will be applied to identify independent immune predictors of disease stage and therapeutic resistance. Receiver operating characteristic analyses will be performed to determine the diagnostic performance of candidate marker combinations in distinguishing precursor states from overt disease, NDMM from RRMM, and low-risk from high-risk SMM. Composite immune signature models will be constructed to evaluate whether $CD14^{+}CD16^{+}$ monocyte phenotypes enhance prognostic resolution beyond established clinical criteria.

A key objective of this study is to determine whether the density of specific surface markers expressed on $CD14^{+}CD16^{+}$ monocytes correlates quantitatively with disease staging and severity across the MGUS → SMM → NDMM → RRMM continuum. Marker density will be assessed using median fluorescence intensity (MFI) or calibrated antibody binding capacity measurements to provide a standardized estimate of surface expression levels rather than simple presence or absence. These quantitative measures will be statistically associated with established clinical staging parameters, including bone marrow plasma cell percentage, serum M-protein concentration, β2-microglobulin levels, free light chain ratio, ISS/R-ISS stage, cytogenetic risk, and in RRMM, number of prior treatment lines and refractory status. Correlation analyses will evaluate whether increasing marker density reflects progressive immune activation, immunosuppression, or antigen exposure as disease advances. Multivariate regression modeling will further determine whether marker density serves as an independent predictor of disease severity after adjustment for conventional clinical variables. A positive association between increasing surface marker density and higher disease stage would support the hypothesis that $CD14^{+}CD16^{+}$ monocytes undergo stage-dependent functional reprogramming and may serve as quantitative immune indicators of myeloma burden and progression.

Expected Outcomes

This study is expected to demonstrate that $CD14^{+}CD16^{+}$ monocytes undergo progressive quantitative expansion and phenotypic remodeling across the MGUS → SMM → NDMM → RRMM continuum, reflecting stage-dependent immune adaptation within the

myeloma microenvironment. We anticipate identifying specific surface marker signatures whose density increases in parallel with clinical severity, including higher plasma cell burden, elevated β2-microglobulin levels, advanced ISS/R-ISS stage, and, in RRMM, treatment refractoriness. Such findings would support the concept that $CD14^{+}CD16^{+}$ monocytes function as recirculating immune sentinels that integrate tumor-derived antigen exposure and inflammatory conditioning during disease evolution.

We further expect to define reproducible combinations of surface markers that discriminate precursor conditions from overt myeloma and distinguish newly diagnosed from relapsed/refractory disease with meaningful sensitivity and specificity. Demonstration of concordance between bone marrow and peripheral blood phenotypes would provide proof-of-concept for minimally invasive blood-based immune biomarker assessment. Statistical modeling is anticipated to show that marker density–based immune signatures enhance prognostic resolution beyond existing clinical staging systems, thereby offering added value for early risk stratification.

Collectively, these outcomes will establish $CD14^{+}CD16^{+}$ monocyte surface phenotypes as clinically relevant biomarkers of disease progression and therapeutic resistance in multiple myeloma, providing a translational foundation for future diagnostic refinement and epitope-level validation using *AMANDUS* and intracellular detection of Apo10, TKTL1, and cytokines like IL-6 using the novel *FlowISiAM* technology.

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Won Fen Wong and **Gin Gin Gan** and **Chin Sum Cheong** includes some antibodies for *AMANDUS and FlowISiAM*, know how transfer and protocol, support regarding selection of specific antibodies against specific biomarkers from INVIGATE, expert assistance in evaluating the results obtained, and integration into the ImmunoTools *FlowISiAM* network.

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