

Macrophage efferocytosis and apoptosis

Adelle C.F. Coster and Mary R. Myerscough

Abstract Efferocytosis, that is, the removal of dead cells by macrophages, is a key process in the growth, regression and resolution of atherosclerotic plaque. Macrophages acquire internalised lipid when they ingest dying cells. Using data from an *in vitro* experiment measuring macrophage population and average ingested lipid per cell, we explore the processes of efferocytosis and apoptosis in this population. We find that the death rate of macrophages increases with time, which, we suggest is effectively due to their age—at the start of the experiment all cells are the same age. We also find that the rate of efferocytosis is not dependent on the average macrophage lipid load. This agrees with the hypothesis that defective efferocytosis may be a driver of atherosclerosis.

1 Introduction

Macrophages play an important role in atherosclerosis. These immune cells clear apoptotic material such as cellular debris and free lipid from inside apoptotic cells by a processes termed efferocytosis. How this process, and the macrophage life cycle itself, interacts with the development of atherosclerotic plaques is still not completely understood. Here we analyse some experimental data [2] from observations of *in vitro* efferocytosis in murine bone marrow-derived macrophages with a view to determining some parsimonious mathematical descriptions for efferocytosis and

Adelle Coster
School of Mathematics and Statistics, University of New South Wales, Sydney NSW 2052, Australia
e-mail: a.coster@unsw.edu.au

Mary Myerscough
School of Mathematics and Statistics, University of Sydney, NSW 2006, Australia
e-mail: mary.myerscough@sydney.edu.au

apoptosis in these cells, by identifying the possible factors implicated in these processes.

2 Experimental Background

Experiments were undertaken in [2] in which primary murine bone marrow-derived macrophages (BMDMs) were differentiated from mouse femur bone marrow hematopoietic stem cells. The BMDMs were cultured in standard growth media and the cells were plated onto slides at approximately 1×10^5 cells per well (wells of two areas in size, 1.8 cm^2 and 2 cm^2 were used), forming a two dimensional layer of cells adhered to the plate surfaces. Cells were incubated for 0, 12, 24, 36, 48, and 60 hours and then fixed with the application of formalin, the cells stained with oil red O (which labels lipid droplets in the cells) and Mayer's hematoxylin (which labels the cells). Microscopy images were obtained for the fixed and stained macrophages. The authors used a machine learning algorithm to determine the number of cells, and the amount of internalised lipid droplet per cell (as an area per cell). In the first of the experiments considered here the cells were incubated in a medium of lipopolysaccharide (LPS) and a stimulant, interferon γ ($\text{IFN}\gamma$), which causes the cells to polarize to the classically activated, M1, state. Cells in the M1 state are associated with acute and chronic inflammation. A second experiment, in which the cells were stimulated with LPS and staurosporin (STPN) was also performed. The application of STPN promotes apoptosis in the cells.

Data from [2] was re-digitized to extract the number of macrophages, and the average lipid per cell as a function of time for the two protocols, see Figure 1.

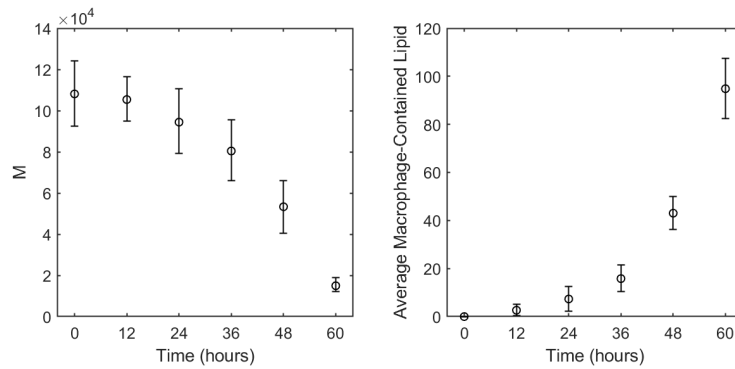


Fig. 1 The macrophage population, M , and the average macrophage-contained lipid load as a function of time, redigitized from [2].

3 Mathematical Modelling

The available data is very sparse, and hence we take a mean field, reductionist modelling approach. The processes that we consider in the model are: replication of the cells, apoptosis (death) of the cells, and efferocytosis, in which apoptotic lipid is ingested by macrophages. This increases the quantity of internalised lipid that they carry.

We note that in the experiments were such that there was no cell immigration or emigration and no other materials in the experimental system apart from the macrophages. Hence the only source of lipid was from the macrophages themselves (largely from their cell membranes). Additionally, all the macrophages were also derived simultaneously from the same source at the start of each experiment and so the cells were all the same age. Given the medium in which the cells were cultured it is expected that the replication rate of the cells was very low.

The state variables we consider for our model of lipid uptake in macrophages are: the live-cell macrophage population, M , the population of apoptotic macrophages (prior to ingestion by efferocytosis), P , the total apoptotic lipid in the system, A_P , and the total macrophage-contained lipid, A_M , which includes any endogenous lipid in the cell membranes. All of these are functions of time, t , taken to be zero at the start of the experiment. Note, as the cells are all the same age, t is also a proxy for the age of the macrophages.

The system of equations for the live and apoptotic cells in the system is as follows:

$$\frac{dM}{dt} = \rho M - fM, \quad \text{and} \quad (1)$$

$$\frac{dP}{dt} = fM - hMP. \quad (2)$$

The corresponding total lipid levels associated with the live and apoptotic cells are given by:

$$\frac{dA_M}{dt} = \rho M a_0 - fA_M + hMA_P, \quad \text{and} \quad (3)$$

$$\frac{dA_P}{dt} = fA_M - hMA_P. \quad (4)$$

Here exponential or Malthusian growth is assumed for cells, with a linear replication rate, ρ , which is assumed to be near zero. Replication increases the macrophage population, M , as well as increasing the total macrophage-contained lipid, A_M , where a_0 is the amount of lipid created to make the cell membranes for the new daughter cell.

The initial conditions are

$$\begin{aligned}
M(0) &= M_0, \\
P(0) &= 0, \\
A_M(0) &= a_0 M_0, \quad \text{and} \\
A_P(0) &= 0.
\end{aligned}$$

The rate of apoptosis or death of the cells is proportional to the macrophage population, M . The function for the rate of apoptosis, $f = f(M, P, A_M, A_P, t)$, may depend on the state variables or time. When the cells die, they move from the live-cell macrophage population, M , to the apoptotic population, P . The internalised lipid in the cell, together with lipid in its cell membrane, is moved from the total macrophage-contained lipid pool, A_M , to the total apoptotic lipid pool, A_P .

Efferocytosis, or the ingestion of apoptotic material by macrophages removes apoptotic cells. It is assumed that this removal is at a rate proportional to the product MP , the probability of a macrophage finding an apoptotic cell. The rate at which this probability is modified, and full ingestion takes place is given by the function $h = h(M, P, A_M, A_P, t)$, which may depend upon the state variables or time. When apoptotic cells are digested they are removed from the system. The apoptotic lipid associated with the cell is removed from the apoptotic lipid pool, and added to the total macrophage-contained lipid.

Thus the average internalised lipid load per (live) macrophage, \bar{L} is given by

$$\bar{L} = \frac{A_M - a_0 M}{M}. \quad (5)$$

3.1 Models for the macrophage and lipid dynamics

Naturally, models that include more degrees of freedom tend to be better able to replicate observations. The functional form of the processes and the resulting dynamics of the state variables have to have appropriate evolutions to match the characteristics of the data however. Here several variants of the rate functions for apoptosis and efferocytosis are explored. We used least-square fitting and constrained the parameters of the models using the data for the macrophage population and the average lipid per live macrophage as functions of time, weighting these two data sets equally. The inferred parameter values for the models are listed in Table 1. The goodness of fit for each of the models is shown in Table 2 – the adjusted R^2 allows for model comparison adjusting for the number of degrees of freedom. Given that the data is extremely sparse, the goodness of fit was poor for all model variants, however the purpose here was not to infer the parameter values with any specificity but rather understand whether the model could reproduce the observed behaviour.

3.1.1 Linear apoptosis and efferocytosis

The data for the macrophage population shows a decrease in this population as a function of time, as might be expected for this experimental system. If we assume the apoptosis rate is linear and constant, $f = \beta$, then the live-cell population is given by an equation for exponential decay, $M = M_0 \exp((\rho - \beta)t)$, where M_0 is the initial macrophage population. If we additionally assume a linear efferocytosis rate function, $h = \eta$ in Equations 1-4, and use least-squares optimisation to fit the parameters to this linear model, the outputs are as shown in Figure 2. It can be seen that the curvature of the plot of the model population as a function of time and the data as a function of time, is at odds and thus a constant apoptosis rate appears to be insufficient to describe the dynamics. The average lipid load is also not well described by this linear model with the model prediction being higher than the data for mid-range values (12 – 36 hours) and very much lower than the last data point (60 hours).

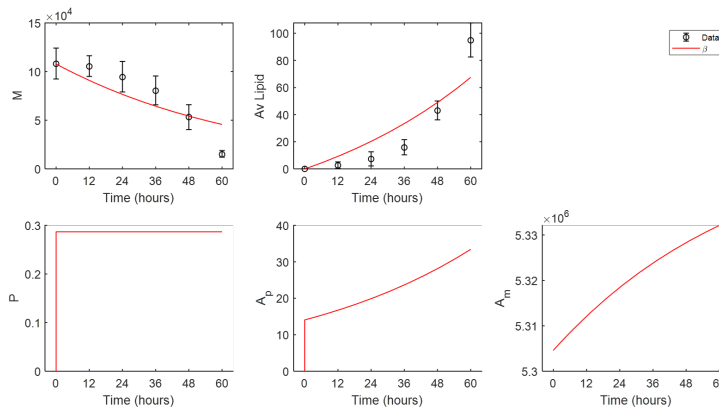


Fig. 2 The macrophage population, M , and the average lipid load per macrophage as a function of time, redigitised from [2]. The solid line indicates the least-squares of the model (Equations 1-4) with with constant apoptosis, $f = \beta$, and efferocytosis rate functions, $h = \eta$. The fit was equally constrained by both data sets. Note the curvature of the macrophage population data and the curve do not match.

3.1.2 Age-driven apoptosis and linear efferocytosis

From the macrophage population data, shown in Figure 1, it appears that the apoptosis rate increases with time. This could be due to the age of the cells (recall that the age of the cells is synchronised in this system) or due to the increasing lipid load that the cells carry as time increases. For this model variant with apoptosis increasing with time (or equivalently age) we heuristically set

$$f = \beta \exp(kt), \quad (6)$$

where β and k are constants, and again assume a linear efferocytosis rate function, $h = \eta$. Optimising the parameters of the system (Equations 1-4) constrained both by the macrophage population and the average lipid load data, the trend for the population decay is captured, (Figure 3). However, the late time dynamics of the average lipid load is underestimated. Therefore, the population dynamics are well-modelled if we assume a death rate that increases with time, but the average lipid load per cell as predicted by the model does not agree well with the data at 48 and 60 hours.

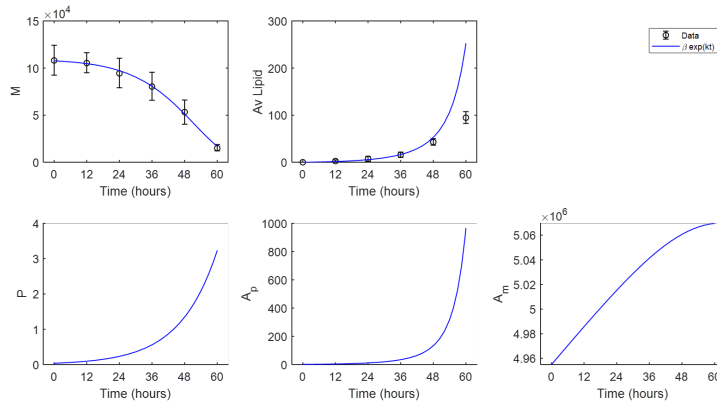


Fig. 3 The macrophage population, M , and the average lipid load per macrophage as a function of time, redigitized from [2]. The solid line indicates the least-squares fit of the model (Equations 1-4) with age-dependent apoptosis and efferocytosis rate function, Equation 6 and constant efferocytosis rate function, $h = \eta$. The fit was equally constrained by both data sets. Note the model trend matches macrophage population data, but overestimates the average lipid load at long times.

As the time (or age) dependent apoptosis rate, Equation 6, captures the macrophage population dynamics it is used all subsequent variants of the model, below.

3.1.3 Age-driven apoptosis and lipid-dependent efferocytosis

The average lipid load at longer times is not well represented by a constant efferocytosis rate, (Figure 3). This could be due to the age of the cells (recall that time can be a proxy for age in this system) or due to the increasing lipid load that the cells carry as time increases. Here we consider the efferocytosis rate to be a decreasing function of lipid load,

$$h = \eta \left(1 - \frac{(A_M)^n}{M^n C^n + (A_M)^n} \right), \quad (7)$$

where η , C , and n are constants. This gives an efferocytosis rate which is maximal at low levels of internalised lipid (which will occur at early times) and then decreases with a change to a minimal rate. The timing and rapidity of transition of efferocytosis as a function of A_M is controlled by the parameters C and n .

The optimised model outputs, constrained both by the macrophage population data and the average lipid per macrophage data are shown in Figure 4 for $n = 2 \dots 10$. The assumption of an age-dependent apoptosis rate function, Equation 6, ensures the macrophage population dynamics are captured. It can be seen, however, that irrespective of the value of n (or the other parameter values), the late time dynamics in the data for the average lipid load cannot be replicated.

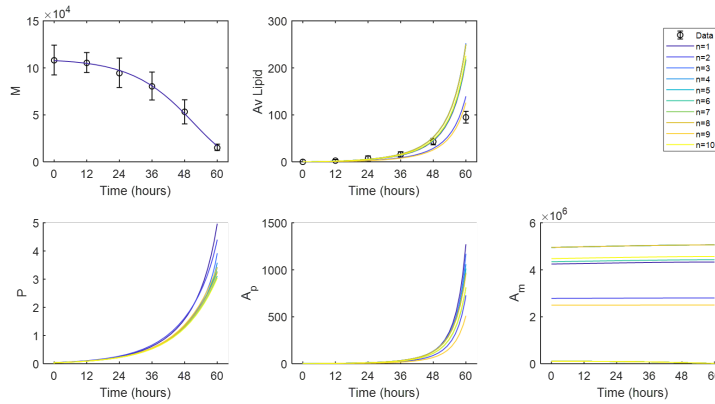


Fig. 4 The macrophage population, M , and the average lipid load per macrophage as a function of time, redigitized from [2]. The solid lines indicates the least-squares of the model (Equations 1-4) with age-dependent apoptosis, Equation 6, for $n = 1 \dots 10$ and lipid-dependent efferocytosis rate function, Equation 7. The fit was equally constrained by both data sets. Note the model trend matches macrophage population data, although it does not match the average lipid levels at long times.

3.1.4 Age-driven apoptosis and efferocytosis

As a lipid-dependent efferocytosis rate appears to be unable to represent the observations, particularly at later times, we test here whether the average lipid content per cell can be explained by efferocytosis becoming less effective as time (and the age of the macrophages) increases. To do this, we make the efferocytosis rate time dependent:

$$h = \eta \exp(-gt). \tag{8}$$

The macrophage dynamics continue to be well described by an age-dependent apoptosis rate, Equation 6, and it can be seen that this, in combination with an age-

dependent efferocytosis rate function, is also able to capture the dynamics of the average lipid load, Figure 5.

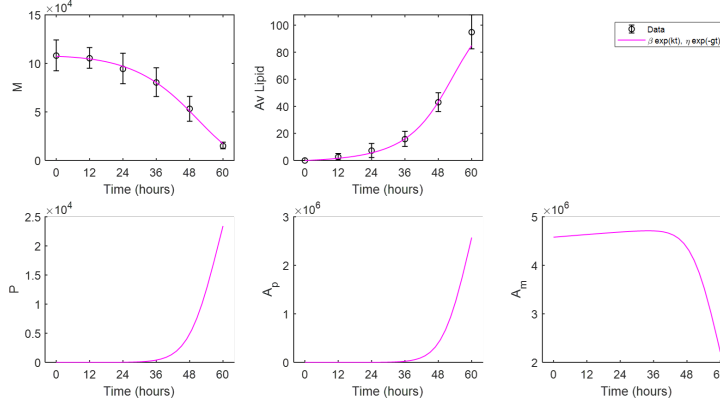


Fig. 5 The macrophage population, M , and the average lipid load per macrophage as a function of time, redigitized from [2]. The solid line indicates the least-squares fit of the model (Equations 1-4) with age-dependent apoptosis and efferocytosis rate functions, Equations 6 and 8. The fit was equally constrained by both data sets. Note the model trends match both the macrophage population data and average lipid timecourse.

f	β	Eqn 6	Eqn 6	Eqn 6
h	η	η	Eqn 7, $n = 2$	Eqn 8
C			639.9 (-9.031e+06, 9.033e+06)	
M_0	1.0960E+05 (9.196e+04, 1.272e+05)	1.0770E+05 (9.838e+04, 1.17e+05)	1.0770E+05 (9.78e+04, 1.177e+05)	1.0790E+05
a_0	45.9 (-2.456e+06, 2.456e+06)	46 (-1694, 1786)	32.88 (-1805, 1871)	45.21
β	0.01545 (-827, 827)	0.001731 (-0.004167, 0.00763)	0.001913 (-0.03905, 0.04288)	0.001575
η	8.6400 (-331.9, 349.2)	0.0457 (0.009066, 0.08225)	0.0214 (-1.552, 1.594)	0.2358 (-0.3742, 0.8458)
k	-	0.07309 (0.004731, 0.1415)	0.07093 (-0.3321, 0.474)	0.07486 (0.01586, 0.1339)
ρ	0.0007022 (-827, 827)	0.0004957 (-0.01336, 0.01436)	0.0007714 (-0.07182, 0.07337)	2.426E-05 (-0.0135, 0.01355)
g				0.2058 (-0.3795, 0.7912)

Table 1 The parameter values for each least-squares fit for the different models, indicated by the apoptosis rate function, f , efferocytosis function, h , and order, n . The 95% confidence interval is indicated in brackets. Both datasets, the macrophage population and the average lipid load as functions of time, were equally weighted to constrained the parameter estimates. Note that the values are not well constrained.

f	h	n	R^2	Adjusted R^2
β	η		0.9056	0.8935
Eqn 6	η	-	0.9721	0.9675
Eqn 6	Eqn 7	2	0.9721	0.9663
Eqn 6	Eqn 7	3	0.9720	0.9662
Eqn 6	Eqn 7	4	0.9720	0.9662
Eqn 6	Eqn 7	5	0.9720	0.9662
Eqn 6	Eqn 7	6	0.9720	0.9662
Eqn 6	Eqn 7	7	0.9720	0.9662
Eqn 6	Eqn 7	8	0.9720	0.9662
Eqn 6	Eqn 7	9	0.9720	0.9662
Eqn 6	Eqn 7	10	0.9720	0.9662
Eqn 6	Eqn 8		0.9837	0.9803

Table 2 The least-squares goodness-of-fit R^2 and Adjusted R^2 , adjusting for the number of model parameters, for each of the different models, indicated by the apoptosis rate function, f , and efferocytosis function, h . Both datasets, the macrophage population and the average lipid load as functions of time, were equally weighted to constrained the parameter estimates.

4 Discussion and Conclusion

In this study, we have identified that apoptosis for a particular population of macrophages cultured *in vitro* depends on age. We also found that death rate depended on age. Since all cells were the same age at the start of the experiment, we can use time t to represent cell age from the start of the experiment. We also found that internalised lipid load in macrophages appears to be best captured by a decreasing efferocytosis rate with time/age. This is in agreement with the theory that atherosclerosis progresses, in part, due to a rise in defective efferocytosis [1, 3].

If macrophages' efferocytic abilities decline with age, then more apoptotic material will be left behind in the tissue. Inside a plaque this will, in due course, undergo secondary necrosis and contribute to a core of free lipid and cellular debris in the plaque. In an *in vitro* experiment, we would expect to see lipid droplets become evident outside of cells. Adding further observations at late time, both between 48 and 60 hours and after 60 hours may give more information about what is occurring and, with suitable modifications to the machine learning protocol, free lipid may be able to be observed and quantified.

It would be interesting to test the hypothesis that the effectiveness of efferocytosis declines with the age of the macrophage. One approach may be to perform similar experiments to [2] using macrophages that have differentiated from monocytes of different ages to explore whether cell age, rather than time as a macrophage, influences efferocytic ability.

Future work would link these models to the other investigations of efferocytosis using beads instead of quantifying internalised lipid [2]. Internalised beads structure the macrophage population in a similar way to accumulated internalised lipids but

could be used to quantify the number of efferocytic interactions explicitly. Such an investigation would have the capacity to explore the processes/parameters which were common across the different experiments and those that were protocol specific. The lipid accumulation experiments in the presence of STPN could also be used for more specificity of the efferocytic parameters, given that STPN is an apoptosis enhancing agent.

References

1. Bäck, M., Yurdagül, A., Tabas, I., Öörmi, K., Kovanen, P.: Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities. *Nature Reviews: Cardiology* **16** (2019)
2. Ford, H.Z., Zeboudj, L., Purvis, G.S., Ten Bokum, A., Zarebski, A.E., Bull, J.A., Byrne, H.M., Myerscough, M.R., Greaves, D.R.: Efferocytosis perpetuates substance accumulation inside macrophage populations. *Proceedings of the Royal Society B: Biological Sciences* **286**(1904) (2019). DOI 10.1098/rspb.2019.0730
3. Tabas, I.: Macrophage death and defective inflammation resolution in atherosclerosis. *Nature Reviews: Immunology* **10** (2010)